Volume XI

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Number 4

Physiological Zoölogy

OCTOBER, 1938

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THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS, U.S.A.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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Physiological Zoölogy is published quarterly by the University of Chicago at the University Press, 5750 Ellis Avenue, Chicago, Ill. The subscription price is $7.50 a year; the price of single copies is $2.25. Orders for service of less than a half-year will be charged at the single-copy rate. Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Hayti, Uruguay, Paraguay, Hawaiian Islands, Philippine Islands, Guam, Samoan Islands, Balearic Islands, Spain, and Venezuela. Postage is charged extra as follows: for Canada and Newfoundland, 15 cents on annual subscriptions (total $7.65), on single copies 4 cents (total $2.29); for all other countries in the Postal Union, 40 cents on annual subscriptions (total $7.90), on single copies 10 cents (total $2.35). Patrons are requested to make all remittances payable to The University of Chicago Press in postal or express money orders or bank drafts.

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For the British Empire, except North America, India, and Australasia: The Cambridge University Press, Fetter Lane, London, E.C. 4, England. Prices of yearly subscriptions and of single copies may be had on application.

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For China: The Commercial Press, Ltd., 211 Honan Road, Shanghai. Yearly subscriptions, $7.50; single copies, $2.25, or their equivalents in Chinese money. Postage extra on yearly subscriptions 40 cents, on single copies 10 cents.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

Applications for permission to quote from this journal should be addressed to The University of Chicago Press, and will be freely granted.

Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

Physiological Zoölogy

Vol. XI OCTOBER 1938 *No. 4*

THE EFFECT OF SUBSTANCES PRODUCED BY CHILOMONAS PARAMECIUM ON RATE OF REPRODUCTION

(Two figures)

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INTRODUCTION

WOODRUFF (1911, 1913) concludes that *Paramecium aurelia*, *P. caudatum*, *Stylonychia pustulata* and *Pleurotricha lanceolata* excrete substances which retard growth.

Robertson (1922, 1923, 1924) maintains that *Enchelys farcimen* and an "infusorian," probably *Colpidium colpoda*, produce substances which accelerate growth. Cutler and Crump (1923, 1924, 1925) repeated Robertson's experiment with *C. colpoda* but found no evidence in support of his contention, nor did the following investigators in similar experiments on various unicellular organisms: Peskett (yeast, 1924, 1925); Greenleaf (*P. caudatum*, *P. aurelia*, *Pleurotricha lanceolata*, and *Stylonychia pustulata*, 1924, 1926); Calkins (*Uroleptus mobilis*, 1926); Myers (*Paramecium caudatum*, 1927); Grimwald (*Colpidium colpoda*, 1928); Chejfec (*Paramecium caudatum*, 1929); Jahn (*Euglena*, 1929); Darby (*Paramecium caudatum*, *Paramecium aurelia*, and *Stylonychia pustulata*, 1930); Di Tomo (*Paramecium caudatum*, 1932); Beers (*Didinium nasutum* and *Stylonychia pustulata*, 1933); Johnson (*Oxytricha* sp., 1933); Phelps (*Glaucoma pyriformis*, 1935, 1936).

However, Yocom (1928) in observations on *Oxytricha* sp. and Petersen (1929) and Dimitrowa (1932) in observations on *P. caudatum*, obtained results which seem to support Robertson's contention.

Chejfec (1929) in accurately controlled experiments demonstrated that the rate of growth in *P. caudatum* is closely correlated with the concentration and the availability of bacteria in the culture medium; and he concluded that the increase in rate of growth observed by Robertson, Yocom, Petersen and Dimitrowa was probably due to increase in the concentration and the availability of the bacteria in the culture medium, not to growth-promoting substance produced by the protozoa in it. Johnson (1933) and Phelps (1935, 1936) on the basis of results obtained in extensive well-planned observations on *Oxytricha* sp. and *Glaucoma pyriformis*, came to essentially the same conclusion.

It is consequently obvious that the evidence in support of Robertson's contention, namely, that protozoa produce a growth-promoting substance, is very much less extensive than that in opposition to it, and that, to obtain worth-while results concerning this contention, variation in the quality, the quantity and the availability of food, as well as variation in other environmental factors, must be accurately controlled.

We have done this in the experiments on *Chilomonas paramecium* considered in the following pages. This organism grows well in sterile solutions which contain only relatively simple chemicals in known concentrations. It is therefore well suited for experiments concerning the problem under consideration.

MATERIAL AND METHODS

The chilomonads used in these experiments were obtained from a culture which had been grown in our laboratory for nearly six years in a sterile solution consisting of $NaC_2H_3O_2$, 150 mg; NH_4Cl, 46 mg; $(NH_4)_2SO_4$, 10 mg; K_2HPO_4, 20 mg; $CaCl_2$, 1 mg; $MgCl_2$, 1 mg; and H_2O, 100 cc, with slight modifications (Mast and Pace, 1933, 1935, 1937; Mast, Pace, and Mast, 1936).[1] This solution will be referred to as the acetate-ammonium culture fluid. The salts in it were Kahlbaum's purest and the water was triple (pyrex glass) distilled (Mast, 1928). Several different methods were used. These

[1] We found that mass cultures in this solution at room temperature die out in 6–10 days but that if some are transferred to fresh culture fluid every second or third day, they live indefinitely.

will be described in detail in the different sections in the following pages. The cultures were sterile thruout in all, and pyrex glass was used thruout in all, in accord with earlier work (Mast and Pace, 1933, p. 328).[2]

RELATION BETWEEN THE NUMBER OF CHILOMONADS IN A GIVEN QUANTITY OF CULTURE FLUID AND THE RATE OF REPRODUCTION

About 50 chilomonads, taken from a vigorous culture growing in sterile acetate-ammonium culture fluid, were put into 0.4 cc fresh sterile culture fluid in a depression on a slide and left 2 or 3 days. Numerous specimens were taken from this culture and passed successively thru 0.4 cc fresh sterile culture fluid in each of 4 depressions. Then 0.4 cc fresh sterile culture fluid was put into each of 16 depressions on sterile slides in petri dishes, and one of the prepared chilomonads into each of 4 of the depressions, 5 into each of 4, 25 into each of 4, and 50 into each of 4, after which all the slides were put into a water-bath at 24.4 $\pm$ 0.2° C. and left 24 hours. Then the chilomonads in each of the depressions were counted and the number recorded. This whole process was repeated eight times. The results obtained are presented in table 1.

This table shows that there were produced in 24 hours, for each chilomonad introduced, an average of 8.75 chilomonads in the depressions which had received 1 chilomonad, 10.50 in those which had received 5 chilomonads, 12.42 in those which had received 25 chilomonads, and 13.78 in those which had received 50 chilomonads. That is that an increase from one chilomonad to 50 in 0.4 cc culture fluid resulted in an increase in the rate of reproduction of 50.6 percent.

This seems to prove conclusively that, within certain limits, the rate of reproduction of *Chilomonas* varies directly with the density of the population. If this is true, then a given number of specimens in a small volume of culture fluid should reproduce more rapidly than the same number in a large volume. This proposition was tested in the following experiments.

RELATION BETWEEN QUANTITY OF CULTURE FLUID AND RATE OF REPRODUCTION

Experiment 1.—Chilomonads were prepared as described above. Then 0.4 cc culture fluid was put into each of 4 depressions on slides in petri dishes, and one of the prepared chilomonads was added to the solution in each depression. This was then repeated with six other quantities of culture fluid. All were then kept in a water-bath at 24.4 $\pm$ 0.2° C. for 24 hours, after which the chilomonads were counted. The whole process, with the exception of a small variation in temperature, was then repeated twelve times. The results obtained are presented in table 2.

This table shows that, as the volume in the depressions decreased, the average number of chilomonads produced in 24 hours from each one introduced, increased from 9.15 in 0.4 cc to 12.98 in 0.006 cc, i.e., more than 40 percent.

[2] A few of our cultures occasionally became turbid with bacteria or infected with mold, which could readily be seen. These were invariably discarded. Some of the others were, from time to time, tested for bacteria by plating on standard nutrient agar. With the exception of an occasional colony or two, we found no indication of bacteria. It is, however, well known that some forms do not grow on this medium. Dr. William T. Burrows, professor of bacteriology in the University of Chicago, made in our laboratory various tests, microscopic as well as cultural, for such forms in our cultures, but found none. It can therefore be concluded that they were actually sterile. An account of these tests will soon appear in *Protoplasma*.

These results therefore strongly support the conclusion reached in the preceding experiment. This conclusion is also supported by the results obtained in the following experiment, in which observations were made on the time at which division in chilomonads in different volumes of culture fluid occurs.

Experiment 2.—One-tenth cc culture fluid was put into each of 12 depressions on slides, and 0.006 cc into each of 12. Then one chilomonad, prepared as described above, was added to the solution in each depression. All the slides were put into a water-bath at 24.4 ± 0.2° C., after which the chilomonads in each depression were counted every half-

TABLE 1

RELATION BETWEEN NUMBER OF CHILOMONADS IN A GIVEN VOLUME OF CULTURE FLUID AND FREQUENCY OF DIVISION

Each day the chilomonads put into all the depressions were taken from those produced the preceding day in depressions 9, 10, 11, or 12. 1st–9th, days; hydrogen-ion concentration, pH 6.8; temperature, 24.4±0.2° C.; Chi., chilomonads.

No. of Chi. Put into Each Depression	Designation of Depressions	Average Number Produced in 24 Hours from Each Chi. Introduced									Total Average	σ
		1st	2d	3d	4th	5th	6th	7th	8th	9th		
1....	1	11.00	8.00	11.00	9.00	8.00	5.00	8.00	6.00	11.00	8.75±0.27	2.45
	2	8.00	12.00	12.00	13.00	5.00	5.00	14.00	8.00	8.00		
	3	10.00	11.00	7.00	11.00	8.00	8.00	8.00	6.00	6.00		
	4	6.00	8.00	12.00	7.00	8.00	6.00	10.00	8.00	13.00		
5....	5	10.40	8.00	8.80	16.80	7.40	12.20	13.60	8.60	9.40	10.50±0.44	3.98
	6	9.00	13.40	13.00	14.60	8.60	7.40	8.80	7.80	14.40		
	7	11.60	6.40	11.20	10.80	9.00	11.80	13.20	8.20	10.60		
	8	12.60	9.00	11.60	12.60	11.60	8.40	7.40	10.20	9.60		
25....	9	10.96	13.08	12.04	14.92	11.12	14.28	12.32	10.16	13.60	12.42±0.23	2.06
	10	11.48	11.88	14.76	14.84	11.44	13.48	11.68	13.92	15.00		
	11	11.16	13.32	10.48	15.56	12.16	14.08	8.08	8.88	14.08		
	12	9.12	14.56	12.72	16.32	11.24	12.88	9.68	9.40	12.68		
50....	13	.....	.....	.....	19.84	11.74	11.52	14.48	14.24	19.68	13.78±0.38	2.83
	14	.....	.....	.....	15.70	10.48	12.44	14.92	11.28	16.46		
	15	.....	.....	.....	13.88	13.78	13.96	7.96	10.96	15.92		
	16	.....	.....	.....	17.22	10.72	11.88	13.08	15.12	13.68		

hour for 6 hours. This was then repeated with other chilomonads. The results obtained are presented in table 3.

This table shows that in the first test, 2 of the 12 chilomonads, each in 0.006 cc culture fluid, had divided in 2 hours, and none of the 12, each in 0.1 cc culture fluid; that in 3 hours, 7 of the former had divided and only 2 of the latter; and that in 6 hours the former produced 33 chilomonads and the latter only 24. It shows also that in the second test the results obtained were essentially the same as in the first.

It can therefore be concluded that the rate of reproduction in *Chilomonas* varies inversely with the volume of culture fluid per individual. The following experiment concerns the extent of this correlation.

RELATION BETWEEN NUMBER OF CHILOMONADS, VOLUME OF CULTURE FLUID AND RATE OF REPRODUCTION

Chilomonads were prepared as in the preceding experiment. Then 0.1 cc culture fluid was put into each of 20 depressions on slides in petri dishes, and 1 chilomonad added to

TABLE 2

RELATION BETWEEN VOLUME OF CULTURE FLUID AND FREQUENCY OF DIVISION IN *Chilomonas*

Each day the chilomonads put into the depressions were taken from those produced the preceding day in depressions 1, 2, 3, or 4. 1st–13th, days; hydrogen-ion concentration, pH 6.8; temperature, 24.4±0.2° C. first to 6th days, 24.7° C. 7th to 10th days.

Volume of Culture Fluid (cc)	Designation of Cultures	Number Produced in 24 Hours from Each Chilomonad Introduced												Total Average
		1st	2d	3d	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	
0.006	1	10	12	16	8	14	8	8	16	16	12	14	12	8	12.98
	2	12	16	8	10	8	8	15	16	16	16	8	8	14	
	3	8	14	8	8	18	14	30	22	8	8	9	14	16	
	4	16	9	8	16	8	8	16	16	31	14	30	8	8	
0.012	5	9	10	8	8	8	9	16	15	10	8	12	8	13	11.38
	6	16	16	8	7	12	8	15	16	15	10	12	8	16	
	7	8	8	12	8	8	6	10	8	25	16	10	11	8	
	8	10	12	8	15	10	16	24	12	16	7	7	6	8	
0.025	9	12	12	13	6	8	8	16	16	15	7	8	10	11	10.04
	10	8	16	8	8	14	5	15	8	6	16	16	5	4	
	11	10	14	4	8	8	8	8	16	15	13	8	8	10	
	12	8	10	8	12	8	8	14	8	8	8	12	8	9	
0.05	13	11	10	14	13	8	4	16	8	15	8	8	8	14	10.61
	14	10	8	4	7	7	8	6	16	16	12	8	8	8	
	15	9	12	8	10	8	8	8	8	12	6	16	4	8	
	16	8	8	12	8	10	8	8	16	8	10	8	8	8	
0.1	17	12	8	16	8	11	8	14	16	16	15	7	16	8	10.54
	18	9	8	8	7	7	16	10	12	10	14	16	12	8	
	19	8	12	4	14	8	8	8	8	15	8	8	8	7	
	20	10	14	7	6	8	8	13	10	14	12	22	8	8	
0.2	21	9	8	8	8	8	12	15	14	15	14	8	8	8	9.69
	22	8	10	8	8	14	8	14	16	8	8	14	8	7	
	23	10	8	9	8	8	8	12	8	16	8	8	14	8	
	24	7	8	8	10	8	6	10	10	14	8	8	8	8	
0.4	25	8	8	4	14	7	8	15	16	11	15	8	8	8	9.15
	26	8	7	8	8	12	6	8	18	16	7	12	4	4	
	27	12	6	11	8	8	7	9	8	12	8	7	8	12	
	28	8	8	10	4	4	11	14	16	10	7	8	12	8	

each of 4 depressions, 5 to each of 4, 10 to each of 4, 25 to each of 4, and 50 to each of 4. All were then put into the water-bath at 24.4 ± 0.2° C. This was repeated three times, once with 0.05 cc culture fluid in each depression, once with 0.025 cc and once with 0.0125 cc. After 24 hours the chilomonads in all the depressions were counted. The whole process was then repeated twelve times. The results are presented in table 4.

By referring to table 4 it will at once be seen that in all the volumes tested, except the lowest, as the number of chilomonads introduced increased, the number produced in 24 hours, per individual introduced, increased to a maximum and then decreased; and that

TABLE 3

RELATION BETWEEN VOLUME OF CULTURE FLUID AND RATE OF REPRODUCTION IN *Chilomonas*

One chilomonad was put into the solution in each depression. Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

Volume of Culture Fluid in Each Depression (cc)	No. of Depressions	Time (in hours) the Chi. Were in the Culture Fluid									
		0	2	2.5	3	3.5	4	4.5	5	5.5	6
		Total No. of Chi. in the Depressions									
0.1	12	12	12	12	14	16	19	21	21	23	24
0.006	12	12	14	16	19	20	23	24	24	25	33
0.1	12	12	.....	.....	13	.....	17	17	19	22	24
0.006	12	12	.....	.....	17	.....	18	22	24	26	31

TABLE 4

RELATION BETWEEN VOLUME OF CULTURE FLUID, NUMBER OF CHILOMONADS, AND RATE OF REPRODUCTION

Each figure represents the average number of chilomonads produced in 24 hours from each one introduced (1 in each of 4 depressions, 5 in each of 4 depressions, etc., on 13 different days). Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

No. of Chi. Put into Each Depression	Volume of Culture Fluid in Each Depression (cc)			
	0.1	0.05	0.025	0.012
	Average No. of Chi. Produced from Each One Introduced			
1	8.89	9.08	9.67	12.19
5	10.12	13.18	13.72	9.20
10	12.28	15.10	15.12	8.80
25	13.63	12.58	10.50	7.41
50	10.34	8.88	7.76	3.76

the optimum for the number introduced was about 25 for 0.1 cc culture fluid, about 10 for 0.05 cc, and about 7 for 0.025 cc, i.e. that the optimum number varies directly with the volume.

If each volume used is divided by the optimum number, so as to obtain the optimum volume per individual, there is obtained 0.004 cc for 0.1 cc volume used, 0.005 cc for 0.05

cc volume used and 0.0036 cc for 0.025 cc volume used. This indicates that the optimum volume of culture fluid for reproduction is approximately 0.004 cc per individual.

The fact that the rate of reproduction increases to maximum as the density of population increases and then decreases, shows that for every number of individuals per given volume below the optimum number there is a number above the optimum which has the same effect on rate of reproduction. This is clearly shown by the results presented in table 4. These show, e.g. that the rate of reproduction with 1 chilomonad in 0.05 cc

TABLE 5

EFFECT OF CHANGES PRODUCED BY *Chilomonas* IN THE CULTURE MEDIUM ON RATE OF REPRODUCTION

Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads

Designation of Experiment	No. of Depression Cultures	Volume (in cc) of Culture Fluid in Each	No. of Chi. Put into Each	Time (in hours) Left	Volume (in cc) of Solution Taken from Each	No. of Chi. Added to Each Volume	Total No. of Chi. Found— After Following Hours: 0	2	3	4	5	6	22	After 22 hr. per Chi. Introduced
A.....	24	0.025	0	2	0.025	1	24	24	26	26	29	33	234	9.75
	24	0.025	5	2	0.025	1	24	26	27	28	39	43	304	12.75
B.....	8	0.1	0	16.5	0.025	1	8	8	8	8	10	10	104	13.00
	8	0.1	1	16.5	0.025	1	8	8	10	12	13	15	117	14.62
	8	0.1	10	16.5	0.025	1	8	10	12	14	14	17	108	13.50
	8	0.1	50	16.5	0.025	1	8	8	8	9	10	13	68	8.50
	8	0.1	200	16.5	0.025	1	8	8	8	9	10	12	48	6.00
C.....	12	0.1	0	16.5	0.025	1	12	12	14	14	16	16	125	10.41
	11	0.1	1	16.5	0.025	1	11	14	14	15	18	18	150	13.33
	12	0.1	10	16.5	0.025	1	12	15	17	18	20	21	128	10.66
	12	0.1	25	16.5	0.025	1	12	16	17	20	21	22	103	8.58
	8	0.1	400	16.5	0.025	1	8	8	6	5	1	0	0	0

culture fluid is practically the same as it is with 50, but much higher with 10. If, then, one finds that the rate of reproduction is the same with a small number of organisms in a given volume of solution as it is with a larger number, it obviously does not prove that the rate of reproduction is not correlated with the density of population.

The question now arises as to what causes the observed correlation between volume of culture per individual or density of population and rate of reproduction.

FACTORS INVOLVED IN THE CORRELATION BETWEEN DENSITY OF POPULATION AND RATE OF REPRODUCTION IN *Chilomonas*

One immediately thinks of changes in the chemical composition of the culture fluid, owing to substances produced or consumed by the organisms, as the cause of the observed correlation between density of population and rate of reproduction; but it is obvious that other factors may be involved, e.g. contact stimulation due to movement of the organisms or mitogenetic rays produced by them.

If it is due to changes in the chemical composition of the culture fluid, the rate of reproduction in culture fluid in which chilomonads have been for some time should differ from that in fresh culture fluid. The following experiments concern this contention. These experiments were performed as follows:

Different numbers of sterile chilomonads were put into different quantities of culture fluid and left different periods of time; then all the chilomonads were removed, and a given amount of the culture fluid was put into each of a number of depressions on slides and one chilomonad (prepared as described above) added to each; then the slides were put into a water-bath at 24.4 ± 0.2° C. and the chilomonads counted at definite intervals. The results obtained are presented in table 5.

This table shows that three experiments were made and that in all reproduction under optimum conditions began markedly sooner and continued at a much higher rate in the culture fluid in which chilomonads had been for some time than in fresh culture fluid. It shows also that as the number of chilomonads which had been in the culture fluid increased, the rate of reproduction increased to a maximum and then decreased to zero. It shows, moreover, that to produce a favorable effect on the rate of reproduction requires a surprisingly short time and that fission is not necessary. This is clearly shown in the results obtained in Experiment A, in which only 5 individuals had been in the culture fluid only 2 hours and none had divided.

Since the differences in rate of reproduction recorded in table 5 were independent of the immediate density of the population in the culture fluid, it is evident that they must have been due to changes in the composition of the culture fluid produced by the chilomonads which had been in it before the records were begun. The results presented in this table therefore prove that *Chilomonas* produces changes in the composition of the culture fluid which promote reproduction if they are moderate and inhibit reproduction if they are excessive; but they do not show whether these changes are due to production or consumption of substances, or both. This problem is considered in the following pages; but before taking it up, we will consider the relation between rate of reproduction and rate of growth under the conditions which obtained in the preceding experiments.

RELATION BETWEEN RATE OF REPRODUCTION AND RATE OF GROWTH IN *Chilomonas*

Under some conditions the rate of reproduction in *Chilomonas* is not closely correlated with rate of growth; e.g. high concentration of carbon dioxide inhibits rate of reproduction much more effectively than rate of growth. This results in very large specimens (Mast and Pace, 1933). The question now arises as to whether or not the changes in the rate of reproduction caused by changes in the culture fluid produced by the action of *Chilomonas* are accompanied by corresponding changes in the rate of growth. If they are, the structure and the size of chilomonads grown in culture fluid which has been acted on by them should be essentially the same as the structure and the size of those grown in fresh culture fluid. This proposition was investigated as follows:

Into each of 12 depressions on slides in petri dishes 0.1 cc culture fluid was put; then 75 chilomonads, taken from a flourishing culture, were added to the solution in each of 4 of the depressions; 400, taken from the same culture, to that in each of 4 others; and none to that in the remaining 4. The petri dishes were then put into the water-bath at 24.4 ± 0.2° C. and left 4 hours. All the chilomonads were then removed, and the solution in the 4 depressions in each of the three groups mixed, respectively. Then 0.025 cc of one of the three mixtures was put into each of 12 depressions in petri dishes; 0.025 cc of

another one of the mixtures into each of 12; and 0.025 cc of the remaining mixture into each of 12. A small chilomonad (produced by recent division) from a flourishing culture, called the "parent culture," was now added to the solution in each of the 36 depressions, and all put into the water-bath at 24.4 ± 0.2° C., after which the chilomonads in each were counted every hour for 6 hours. Then all the chilomonads in the 36 depressions and 10 taken from the parent culture were fixed and stained for starch and fat, first in Lugol solution and then in Sudan III and sketched (Fig. 1). The 10 chilomonads

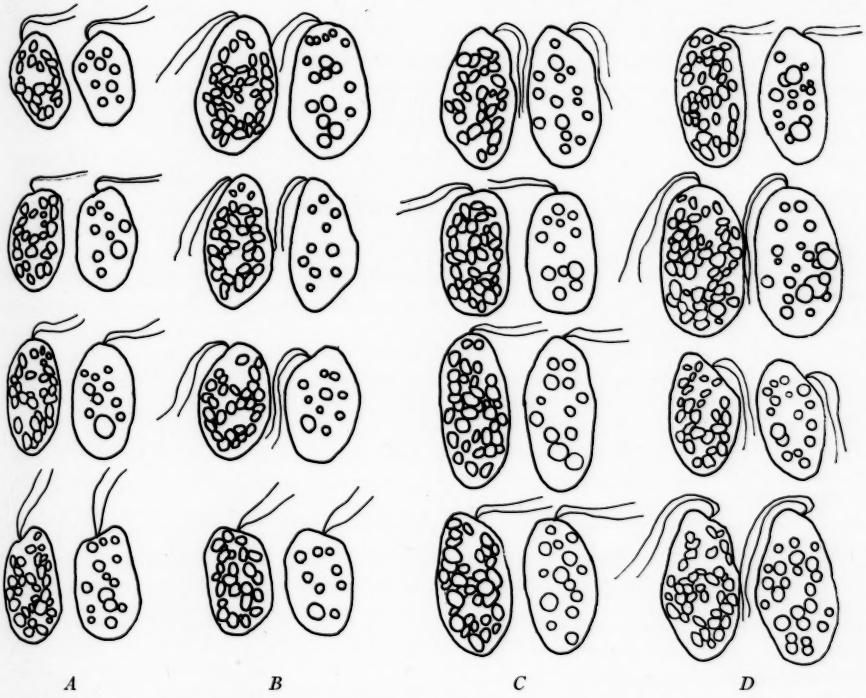

FIG. 1.—Camera outlines showing the effect produced on size and on starch and fat content in *Chilomonas* by changes produced in the culture fluid by it. *A*, small specimens used to start three depression-slide cultures—*B*, *C*, and *D*. *B*, chilomonads taken at random from 7-hour-old cultures in fresh acetate-ammonium culture fluid; *C*, chilomonads taken at random from 7-hour-old cultures in acetate-ammonium culture fluid in which 75 chilomonads per 0.1 cc had been for 4 hours; *D*, chilomonads taken at random from 7-hour-old cultures in acetate-ammonium culture fluid in which 400 chilomonads per 0.1 cc had been for 4 hours. Each pair of large outlines consists of two made of the same specimen. The small outlines in the large outline at the left represent starch grains; those in that at the right represent fat droplets. Hydrogen-ion concentration, pH 6.8; temperature, 24.4 ± 0.2° C.

taken from the parent culture were, in size and in other respects, like those put into the culture fluid in the 36 depressions at the beginning of the experiment.

In the chilomonads in the fresh culture fluid, the first division occurred during the fourth hour in it (Table 6); in those in the culture fluid in which 75 chilomonads had been

for 4 hours, it occurred during the second hour; and in those in the culture fluid in which 400 chilomonads had been for 4 hours, it did not occur until the sixth hour.

In the first of these solutions 8 of the 12 specimens divided in 7 hours; in the second, 12; and in the third, 7. It is consequently evident that in the culture fluid in which 75 chilomonads had been for 4 hours, fission began markedly sooner and continued at shorter intervals than in the fresh culture fluid; and that in the culture fluid in which 400 chilomonads had been for 4 hours, it began considerably later and continued at longer intervals than in the fresh culture fluid. There was, then, a very definite difference in the rate of reproduction in these culture fluids.

If, now, the sketches (Fig. 1) of specimens taken from them are compared, it will be seen that there was no essential difference in the starch and the fat content, but that

TABLE 6

RELATION BETWEEN INCREASE IN RATE OF REPRODUCTION AND INCREASE IN RATE OF GROWTH CAUSED BY CHANGES IN THE CULTURE FLUID PRODUCED BY *Chilomonas*

Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; "ac-am," acetate-ammonium culture fluid; Chi., chilomonads.

Culture Fluid Used	Total No. of Chi. in 12 Depression Cultures after Following Hours:					Volume in Cμ of Chi. Produced in 7 Hr.	
	0	4	5	6	7	Total	Av. per Individual
Fresh ac-am	12	13	15	18	20	46,700	2,333
Ac-am in which 75 chi. per 0.1 cc had been 4 hr.	12	17	18	23	24	76,872	3,202
Ac-am in which 400 chi. per 0.1 cc had been 4 hr.	12	12	12	17	19	53,048	2,792
Volume of 12 chi. used to start cultures						17,940	1,494

those from the culture fluids which had had respectively 75 and 400 chilomonads were somewhat larger than those from the fresh culture fluid, and much larger than those taken from the parent culture.

The volume of each of the chilomonads sketched was calculated by means of the length and the width obtained from measurements made on the sketches and on the assumption that they are ellipsoidal in form.[3] The results obtained are presented in table 6. These results indicate that the total volume of the chilomonads produced in the 12 depressions which contained fresh culture fluid was 46,700 cμ (an average of 2,333 cμ per chilomonad), that the total volume of those produced in the culture fluid which had had 75 chilomonads for 4 hours was 76,872 cμ (an average of 3,202 cμ per chilomonad), that the total volume of those produced in the culture fluid which had had 400 chilomonads for 4 hours was 53,048 cμ (an average of 2,792 cμ per chilomonad), and that the volume of those taken from the parent culture to start each of the three sets of cultures referred to above was 13,940 cμ (an average of only 1,494 cμ per chilomonad).

[3] *Chilomonas* is slightly flattened. The camera outlines show the maximum width. This width was used in calculating the volumes presented. These are, therefore, somewhat too large.

It is consequently evident that the average volume per chilomonad increased considerably in all three culture fluids, i.e. from 1,494 cμ to 2,333, 3,202, and 2,792 cμ, respectively. The fact that it increased more in the culture fluid in which 75 chilomonads had been for 4 hours than in the fresh culture fluid shows that the change produced by the 75 chilomonads in the culture fluid caused, not only an increase in the rate of reproduction, but also an increase in the rate of growth and that the increase in the rate of growth was, in fact, considerably greater than the increase in the rate of reproduction. It can consequently be concluded that the change in the culture fluid produced by *Chilomonas* acts primarily on the rate of growth and secondarily on the rate of reproduction.

THE NATURE OF THE CHANGE IN THE CULTURE FLUID PRODUCED BY *Chilomonas*

About 1,000 sterile chilomonads were put into 1.5 cc sterile culture fluid in a columbia dish in a petri dish. These multiplied and soon became very abundant but all died in about 6 days. Then some of the solution in the dish was put into each of five small pyrex glass tubes and the same amount of fresh culture fluid into each of two. One of the former and one of the latter were left at room temperature. All the rest were suspended in water in a beaker over a gas flame and left until it began to boil; then one of those containing old culture fluid was removed, 30 minutes later another of these and the one containing fresh culture fluid, 30 minutes later another containing old culture fluid and 30 minutes later still another. After the tubes had cooled to room temperature, 0.025 cc of the solution in each of these tubes and those left at room temperature was put, respectively, into each of 12 depressions on slides in petri dishes; then a small chilomonad from a vigorous culture was passed through four separate portions of fresh culture fluid and added to each, after which all were put into the water-bath at 24.2 ± 0.2° C. and examined from time to time for 22 hours. The results obtained are presented in table 7. This experiment was repeated. The results obtained are practically the same as those presented.

Table 7 shows that there were produced in the 12 depressions with fresh culture fluid, not heated, 8 individuals in 8 hours and 142 in 22 hours and practically the same number in the fresh culture after it had been at 100° 30 minutes; and that in the old culture fluid, not heated, none were produced in 8 hours and only 34 in 22 hours, but that in this fluid after it had been at 100° for 60 minutes there were produced 9 in 8 hours and 140 in 22 hours, i.e. very nearly the same number as in the fresh culture fluid.

The fact that subjecting the old culture fluid to 100° for 60 minutes caused an increase in the rate of reproduction from nearly zero to practically that which obtains in fresh culture fluid demonstrates conclusively that the reduction in rate of reproduction with increase in the age of the culture is not due to consumption of substances by the organisms in the culture fluid and that it must therefore be due to the production of substance by them; and it demonstrates, moreover, that the substance produced is heat-labile. The results presented have, however, no direct bearing on the nature of the changes produced by the organisms in the culture fluid, which cause an increase in the rate of reproduction. The following experiments concern this problem.

These experiments were performed in essentially the same way as the preceding except that, in place of leaving the chilomonads in the culture fluid until the culture died out, they were left only 4 hours. But in some of these experiments there were 35 chilomonads

per 0.1 cc acetate-ammonium culture fluid and in others, 150. The results obtained in the former are presented in table 8, and those obtained in the latter in table 9.

Table 8 shows that the average time required for division in fresh acetate-ammonium culture fluid was, in the two experiments made, respectively 6.75 and 6.83 hours, and that subjecting this fluid to 100° for 30 minutes did not significantly change the time

TABLE 7

THE EFFECT OF TEMPERATURE ON THE MAXIMUM CHANGE PRODUCED BY *Chilomonas* IN CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "old ac-am fluid," culture fluid in which a culture of chilomonads had been until it died out; Chi., chilomonads; temperature, 24.2 ±0.2° C.; hydrogen-ion concentration, pH 6.8.

Culture Fluid Used	Depression Cultures										
	No. of Cultures	Quantity of Fluid (cc) in Each	No. of Chi. Added to Each	Total No. of Chi. after Following Hours:							Total No. per Individual Introduced	Time (in hours) to First Division
				1	4	5	6	7	8	22		
Fresh ac-am fluid at room temperature	12	0.025	1	12	12	13	16	18	20	154	12.83	7.41
Fresh ac-am fluid after having been at 100° for 30 min.	12	0.025	1	12	12	13	15	18	21	155	12.91	7.41
Old ac-am fluid at room temperature	12	0.025	1	12	12	12	12	12	12	46	3.83	No division in 8 hr.
Old ac-am fluid after having been at 100° for 10 sec.	12	0.025	1	12	12	12	13	15	18	118	9.83	7.88
Same, except at 100° for 30 min.	12	0.025	1	12	12	15	19	20	24	142	11.83	6.50
Same, except at 100° for 60 min.	12	0.025	1	12	12	14	15	18	21	152	12.66	7.33
Same, except at 100° for 90 min.	12	0.025	1	12	12	13	15	17	20	150	12.50	7.58

required; but that, after 35 chilomonads per 0.1 cc had been in this fluid 4 hours, the average time was reduced to 6.16 and 6.33 hours, respectively, and that, as the time this fluid was subjected to 100° increased, the average time for division increased, reaching, after 30 minutes at 100°, respectively 6.91 and 6.75 hours in the two experiments, i.e. practically the same as in the fresh fluid.

This demonstrates conclusively that the change in the culture fluid produced by the chilomonads, which accelerates rate of reproduction, is not due to consumption of any-

thing in the fluid by the chilomonads and that it therefore must be due to a substance produced by them. The fact that temperature destroys this substance just as it does that which inhibits reproduction, indicates that these substances have the same properties; and the fact that if chilomonads are left in the culture fluid only a short time, the rate of reproduction is accelerated, whereas if they are left a long time it is inhibited, indicates that the effect of the substance produced by *Chilomonas* depends upon the concentration, a weak solution causing acceleration and a strong solution inhibition.

These conclusions are supported by the fact that it requires much longer exposure to high temperature to destroy the inhibiting substance than the accelerating, as will be

TABLE 8

THE EFFECT OF TEMPERATURE ON SMALL CHANGES PRODUCED BY *Chilomonas* ON CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "young ac-am fluid," culture fluid in which 35 chilomonads per 0.1 cc had been for 4 hours; temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8.

Culture Fluid Used	Average Time (in hours) for 12, Each in 0.025 cc Fluid			
	To First Division	First to Second Division	To First Division	First to Second Division
	Experiment A		Experiment B	
Fresh ac-am fluid at room temperature	6.75	6.18	6.83	5.12
Same, except at 100° for 15 min.	7.08	6.00	6.75	5.08
Young ac-am fluid at room temperature	6.16	4.87	6.33	4.75
Same, except at 100° for 10 sec.	6.33	5.50	6.41	5.08
Same, except at 100° for 15 min.	6.41	5.83	6.66	5.20
Same, except at 100° for 30 min.	6.91	5.64	6.75	5.25
Same, except at 100° for 60 min.	6.66	5.45	6.66	5.20

clearly seen by comparing tables 7 and 8. They are also supported by the results presented in table 9.

This table shows that after 150 chilomonads per 0.1 cc had been in the culture fluid for 4 hours there was, in both experiments made, an increase in the time required for division, i.e. from 5.75 to 6.00 hours in the one and from 6.50 to 7.15 hours in the other, but that after this fluid had been subjected to 100° the time required for division decreased to a minimum (as the time at 100° increased) and then increased to normal.

This indicates that after 150 chilomonads per 0.1 cc have been in the culture fluid 4 hours the substance produced by them is sufficiently concentrated to cause retardation in the rate of reproduction; whereas after only 35 per 0.1 cc have been in the fluid for 4 hours (Table 8) it is so weak that it causes acceleration, that the marked increase in the rate of reproduction after the fluid has been subjected to 100° is due to destruction of a portion of the substance and consequent decrease in concentration and that the increase in the rate of reproduction to normal after longer subjection to 100° is due to destruction of all of the substance.

The results presented show then that if culture fluid in which *Chilomonas* has died out and in which the concentration of X is maximum is subjected to temperature of 100° and then cooled, the rate of reproduction in it increases from zero to maximum, as the time at 100° increases, and then decreases to that which obtains in fresh culture fluid. This indicates that X at 100° gradually decreases from lethal concentration thru optimum concentration to zero, as the time increases.

The conclusions reached are also supported by the results obtained with old culture fluid (fluid in which the chilomonads had died out) mixed with fresh culture fluid. The methods used in the experiment made with this mixture were the same as those used in

TABLE 9

EFFECT OF TEMPERATURE ON MEDIUM CHANGES PRODUCED BY *Chilomonas* ON CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "medium ac-am fluid," culture fluid in which 150 chilomonads per 0.1 cc had been for 4 hours; temperature, 24.4 ± 0.2° C.; hydrogen-ion concentration, pH 6.8.

Culture Fluid Used	Average Time (in hours) for 12 Chi., Each in 0.025 cc Fluid			
	To First Division	First to Second Division	To First Division	First to Second Division
	Experiment A		Experiment B	
Fresh ac-am fluid at room temperature	5.75	4.96	6.50	6.00
Fresh ac-am fluid after having been at 100° for 15 min.	5.83	4.83	6.33	6.12
Medium ac-am fluid at room temperature	6.00	5.83	7.16	6.77
Same, except at 100° for 10 sec.	4.91	4.75	5.58	6.00
Same, except at 100° for 15 min.	4.25	4.75	5.25	5.58
Same, except at 100° for 30 min.	4.75	4.50	5.91	5.62
Same, except at 100° for 60 min.	5.75	4.75	6.41	6.12

the preceding experiments. The average time required for division of 12 chilomonads, each in 0.025 cc culture fluid, was 6.83 hours for fresh fluid, more than 9 hours for old fluid, 6.83 hours for a mixture of old fluid (3 parts) and fresh fluid (1 part), 5.25 hours for old fluid (1 part) and fresh fluid (1 part), and 6.33 hours for old fluid (1 part) and fresh fluid (3 parts).

This demonstrates conclusively that the effect on rate of reproduction of the substance produced by *Chilomonas* is specifically correlated with its concentration, the lower concentrations causing acceleration and the higher retardation.

In the following pages we shall call this substance "X."

MINIMUM AND OPTIMUM CONCENTRATIONS IN THE CULTURE FLUID OF SUBSTANCE (X) PRODUCED BY *Chilomonas*, AS INDICATED BY ITS EFFECT ON RATE OF REPRODUCTION

In ascertaining the minimum and the optimum concentrations of substance X in its effect on the rate of reproduction in *Chilomonas*, different numbers of chilomonads from a vigorous culture were put, respectively, into 0.1 cc acetate-ammonium culture fluid

in depressions on slides and left 4 hours, after which all were removed. Then the rate of reproduction in the fluid in the different depressions was ascertained, as described in the preceding sections. This experiment was repeated twice. The results obtained are presented in table 10.

This table shows that, as the number of chilomonads per 0.1 cc culture fluid increased, the average time required for division of 12 specimens decreased from 6.6 hours in fresh culture fluid to a minimum of 4.87 in culture fluid in which 75 chilomonads per 0.1 cc had been for 4 hours and then increased to 7.36 hours; and that there was a slight reduction in the culture fluid in which only 1 chilomonad had been for 4 hours. It shows

TABLE 10

THE MINIMUM AND THE OPTIMUM CONCENTRATIONS OF SUBSTANCE X IN THE CULTURE FLUID, AS INDICATED BY ITS EFFECT ON THE RATE OF REPRODUCTION IN *Chilomonas paramecium*

"Ac-am," acetate-ammonium culture fluid; Chi., chilomonads; temperature, 24.4 ± 0.2° C.; hydrogen-ion concentration, pH 6.8.

Culture Fluid Used	Time (in hours) to First Division			
	Average for 12 Chi., Each in 0.025 cc Fluid in Each of the Following Experiments:			Total Average
	A	B	C	
Fresh ac-am fluid	7.16	6.16	6.66	6.66
Ac-am fluid in which 1 chi. per 0.1 cc had been for 4 hr.	7.16	.....	6.00	6.58
Same, except 10 chi.	6.66	5.66	.....	6.16
Same, except 20 chi.	6.41	5.75	.....	6.08
Same, except 50 chi.	6.41	5.41	.....	5.91
Same, except 75 chi.	.....	5.08	4.66	4.87
Same, except 100 chi.	5.66	5.25	5.75	5.55
Same, except 150 chi.	.....	5.50	.....	5.50
Same, except 200 chi.	7.00	6.75	8.33	7.36

however that this reduction occurred in only one of the two experiments made. But in two additional experiments the average time required for the first divisions was, respectively, 8.25 and 6.83 hours in fresh culture fluid and 7.41 and 6.66 hours in culture fluid in which 1 chilomonad had been for 4 hours. There was consequently a definite reduction in three of the four tests made.

It can therefore be concluded that the concentration of substance X produced by 1 chilomonad in 0.1 cc culture in 4 hours is high enough to have a slight accelerating effect on the rate of reproduction, that the concentration of this substance produced by 75 chilomonads in 0.1 cc culture fluid for 4 hours is optimum in its effect on rate of reproduction and that the concentration of 200 chilomonads in 0.1 cc for 4 hours causes retardation in the rate of reproduction.

If it is assumed that a chilomonad produces in 4 hours, substance X equal in amount to one-tenth its volume (which would seem to be a very liberal estimate) the concentration of this substance in the culture fluids used can readily be calculated.

The average volume of each chilomonad used was approximately 1,230 $c\mu$. Each chil-

omonad would then produce approximately 123 $c\mu$ in 4 hours. The volume of the culture fluid in which substance X was produced equals 10^{12} $c\mu$. The concentration of substance X in the culture fluid in which 1 chilomonad had been for 4 hours (i.e. the minimum effective concentration) would therefore be 123 parts of X in 10^{11} parts of culture fluid, or approximately 1 part in 800,000,000; the concentration of this substance in the culture fluid in which 75 chilomonads had been for 4 hours (i.e. the optimum concentration) would be about 1 part in 10,000,000; and the concentration of the substance in the culture fluid in which 200 chilomonads had been for 4 hours (i.e. the minimum concentration which causes retardation in the rate of reproduction) would be approximately 1 part in 4,000,000.

While it is obvious that all these results are crude approximations, they nevertheless show that substance X is effective in extremely low concentrations in the culture fluid.

RELATION BETWEEN CONCENTRATION IN PROTOPLASM OF SUBSTANCE X PRODUCED BY *Chilomonas* AND RATE OF REPRODUCTION

In the preceding sections it was demonstrated that the rate of reproduction of *Chilomonas* is closely correlated with the concentration of X in the culture fluid, but the results show that there was great variation in the rate of reproduction in experiments made on different days with culture fluid in which the concentration of X was the same. This becomes obvious if the results presented in the preceding tables are compared. Tables 7, 9, and 10 for example, show that the time required for the first division after the chilomonads had been transferred to fresh acetate-ammonium solution in the different experiments made varied from 5.75 to 7.41 hours.

The chilomonads used in all the experiments were taken from vigorous cultures and were carefully selected for size. The temperature and the culture fluid were practically the same. What, then, was the cause of the variation in the rate of reproduction observed? The following experiments concern this question.

Experiment 1.—Four groups of 12 isolation cultures each were set up in depressions on slides. Each culture contained 0.025 cc acetate-ammonium culture fluid and 1 small chilomonad, selected for size and content from a young flourishing culture.[4] All the cultures were kept in the water-bath at 24.4 $\pm$ 0.1° C. and examined every hour until each chilomonad had divided twice. Then the average time required for the first and for the second division, respectively, was calculated for the 12 chilomonads in each group. It was found that the first division required respectively 6.20, 6.10, 6.00, and 6.25 hours in the four groups and the second division, respectively 5.25, 5.16, 5.45, and 5.25 hours.

These results show that if several groups of chilomonads are selected from a given culture at the same time, the average rate of reproduction in the different groups is fairly uniform; and they indicate that the excessive variation observed in the preceding experiments must in some way have been correlated with the fact that the chilomonads used were taken from different cultures, despite the fact that all these cultures were in a flourishing condition.

The results presented also show that, after the chilomonads have been transferred from an acetate-ammonium culture fluid in which they had grown for some time and which consequently contains considerable substance X, to fresh acetate-ammonium culture

[4] Before the chilomonads were put into the isolation cultures, they were passed successively thru each of 4 portions of sterile fresh culture fluid in depressions on slides.

fluid which contains no X, the first division requires much more time than the second division.

This is what would be expected if the chilomonads contained, in their protoplasm, X in supraoptimum concentration when they were transferred from the old to the fresh culture fluid (as was probably true) and if, after they were transferred, some of X diffused out so as to decrease its concentration in the protoplasm and increase it in the culture fluid, thus making it more nearly optimum in both, and consequently causing an increase in the rate of multiplication.

Experiment. 2.—Approximately 500 chilomonads were taken from a culture which was 3 days old and put into a depression on a slide which contained 0.1 cc acetate-ammonium culture fluid. This culture was then put into the water-bath at 24.5 ± 0.2° C. and left 24 hours; then 500 of the chilomonads from this culture were transferred to each of two depressions, each containing 0.1 cc culture fluid, and left 24 hours; after which 500 were transferred from one of these depressions to each of two others, each containing 0.1 cc culture fluid. This was repeated 24 hours later, resulting in three cultures which were precisely the same except that the first was 1 day older than the second, and the second 1 day older than the third. These three cultures were left in the water-bath 24 hours, then 12 small chilomonads which had recently divided were selected from those in each culture, washed as described above, and put respectively into 0.025 cc culture fluid in depressions on slides in petri dishes in the water-bath, after which they were examined every hour and the average time required for division in each group calculated.

It was found that those from the one-day-old culture required an average of 6.08 hours; those from the two-day-old culture, 7.75 hours; and those from the three-day-old culture, 7.91 hours.

This experiment was repeated several days later and the averages obtained were 6.25, 7.08, and 7.33 hours, respectively.

This shows that the rate of reproduction in chilomonads which have been transferred to fresh culture fluid is closely correlated with the age of the culture from which they are taken.

We have demonstrated that the concentration in the culture fluid of substance X varies directly with the age of the culture and the density of the population. It was consequently much more concentrated in the three-day-old than in the one-day-old culture, and the protoplasm in the former doubtless contained much more than that in the latter. The concentration in all three cultures was supraoptimum, for in the youngest culture it equaled the amount produced in 0.1 cc by 500 chilomonads in 24 hours or approximately 28,000 chilomonad-hours per 0.1 cc; whereas the optimum, as shown above, is approximately only 300 chilomonad-hours per 0.1 cc. In the oldest culture it was, however, very much higher, i.e. over 2,000,000 chilomonad-hours per 0.1 cc.

We would therefore expect the time required for division in fresh culture fluid to be much shorter in the former than in the latter, which is precisely what was observed.

The variation in the rate of reproduction in fresh culture fluid with the age of the culture from which the chilomonads have been taken appears therefore to be due to differences in the amount of X in the protoplasm carried over in the transfer. This conclusion is strongly supported by the results obtained in the following experiment.

Experiment 3.—Five hundred chilomonads were taken from a vigorous culture and put into 0.1 cc fresh acetate-ammonium culture fluid in a depression on a slide in the water-bath at 24.5 ± 0.2° C. Four days later 500 more were taken from a similar culture

and treated in the same way and 1 day later one from another similar culture. One day after this, 12 small individuals which had recently divided were taken from each of these three depression cultures, washed as in the preceding experiments, and put separately into 0.025 cc culture fluid in each of 36 depressions on slides in petri dishes. This resulted in 12 isolation cultures, each of which contained 1 chilomonad from a six-day-old culture containing very many specimens, 12 which contained chilomonads from a two-day-old culture containing a moderate number, and 12 which contained chilomonads from a two-day-old culture containing only a few (about 64). The concentration of X in the protoplasm of the chilomonads in the first group of cultures was therefore "strong," that in the protoplasm of those in the second group "medium," and that in the protoplasm of those in the third group "weak." All these cultures were kept in the water-bath and examined hourly.

After the first division of the chilomonad in each of these cultures, one was removed and put into 0.025 cc fresh culture fluid in a clean depression on a slide in a petri dish, and the other was left in the old culture fluid. After all had divided, there were therefore 36 isolation cultures which contained one-day-old culture fluid and 36 which contained fresh culture fluid. The former were designated "A," and the latter "B." All were put into the water-bath and then examined hourly. In A, 1 chilomonad was removed and discarded after each division, and the culture fluid removed (only a small amount) with it was replaced with fresh culture fluid. In B, 1 chilomonad was removed after each division and put into 0.025 cc fresh culture fluid in a clean depression, and the other one discarded. The results obtained are presented in table 11 and figure 2.

This table shows that the time required for the first division varied greatly, i.e. from 6.08 to 10.10 hours, and that it varied inversely with the concentration of X in the protoplasm of the chilomonads.[5] It also shows that in both A and B, as the time increased, the rate of reproduction under all three conditions increased to a maximum and then decreased but that the rate of decrease was very much more rapid and the maximum rate considerably higher in the chilomonads in which the concentration of X was "strong" than in those in which it was "weak" and that the maximum rate of reproduction was, in general, probably reached a little sooner in A than in B. It shows however, that at the close of the experiment the rate was very nearly the same under the three conditions but that it was a little higher in A than in B. The table indicates that in the chilomonads in which the concentration of X was weakest there was a slight decrease in the rate of reproduction before the increase to a maximum occurred and that this decrease was considerably higher in B than in A. All these results become much more evident by referring to and comparing the curves in figure 2. What do they signify?

The concentration of substance X in the culture fluid from which the chilomonads used in this experiment were taken was approximately 504 chilomonad-hours per 0.1 cc in "weak," 252,000 in "medium," and more than 1,000,000,000 in "strong." It was therefore supraoptimum in all, for the optimum is only 300 (Table 10); and the concentration in the protoplasm of the chilomonads was probably somewhat higher than that in the culture fluid. In the transfer of the chilomonads from the old culture fluid to fresh culture fluid the X in the protoplasm was of course also transferred; one would therefore expect the rate of reproduction in the fresh culture fluid immediately after the chilo-

[5] This experiment was in part repeated twice. In one test the average time required for the first division was 7 hours with chilomonads from a young culture and 10.20 with chilomonads from an old culture, and in the other it was 6.41 and 10.80 hours, respectively.

monads were transferred to it to vary inversely with the concentration of X in their protoplasm in accord with the results obtained. And since, after the transfer, X doubtless (owing to difference in concentration) diffused out of the protoplasm and became weaker and weaker, reaching optimum concentration and then suboptimum, one would expect that, as the time in fresh culture fluid increased, the rate of reproduction under all three conditions investigated would increase to a maximum and then decrease, which

TABLE 11

THE RELATION BETWEEN THE CONCENTRATION OF SUBSTANCE X IN THE PROTOPLASM OF *Chilomonas paramecium* AND THE RATE OF REPRODUCTION IN FRESH ACETATE-AMMONIUM CULTURE FLUID

Concentration of X in protoplasm: weak, chilomonads taken from a culture started with 1 chilomonad in 0.1 cc acetate-ammonium culture fluid and left 2 days; medium, chilomonads taken from a culture started with 500 in 0.1 cc acetate-ammonium fluid and left 2 days; strong, chilomonads taken from a culture started with 500 in 0.1 cc and left 6 days. Temperature, 24.5 ± 0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

In experiment A, after each division, one of the chilomonads was removed from the culture and discarded. In experiment B, after each division, one of the chilomonads was put into a clean depression containing 0.025 cc fresh acetate-ammonium culture fluid and the other one discarded.

Concentration of Substance X in Protoplasm of Chi.	Average Time (in hours) for 12 Chi. in Isolation Cultures							
	To First Division	First to Second Division	Second to Third Division	Third to Fourth Division	Fourth to Fifth Division	Fifth to Sixth Division	Sixth to Seventh Division	Seventh to Eighth Division
	A							
Weak	6.08	6.00	6.33	5.08	4.83	5.08	5.66	5.90
Medium	7.60	5.66	5.58	5.00	5.00	4.45	4.66	6.00?
Strong	10.10	5.90	4.33	4.87	4.37	4.28	5.00	5.80?
	B							
Weak	6.08	6.25	6.75	5.83	5.50	5.41	5.83	.....
Medium	7.60	4.70	5.50	5.90	5.20	5.30	6.66	6.40?
Strong	10.10	5.90	5.00	5.22	4.50	5.00	5.50	6.50?

is precisely what was observed; and one would expect the maximum rate to be reached a little later and the rate at the close of the experiment to be a little higher in A than in B, for the concentration of X was, after the first division, continuously a little higher in A than in B, owing to the fact that culture fluid was not changed in A. The latter expectation is in accord with what was observed but the former probably is not, although there is some doubt concerning this.

The correspondence between expected and observed strongly supports the conclusions reached from the results obtained in the preceding experiment, namely, that the rate of reproduction in *Chilomonas* is closely correlated with the concentration of X in the protoplasm and that the variation in the rate of reproduction in fresh culture fluid

observed in the experiments described in the preceding sections of this paper was due to variation in the concentration of X in the culture fluid from which the chilomonads used were taken.

But why the rate of reproduction in the experiment now under consideration increased much more rapidly in chilomonads transferred from old than in those transferred from young culture fluid to fresh culture fluid and why the maximum rate was higher in the former than in the latter and why there was at first decrease in the latter and none in the former and why this decrease was greater in B than in A is not clear. There are, of course, various other factors besides X involved in the control of the rate of reproduction in *Chilomonas*, and their action doubtless accounts for these results.

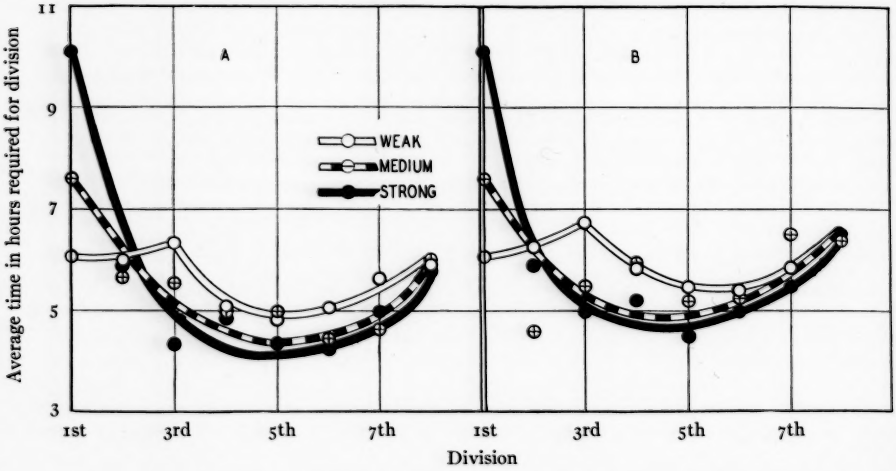

FIG. 2.—The relation between the concentration of substance X in the protoplasm of *Chilomonas paramecium* and the rate of reproduction in fresh acetate-ammonium culture fluid. Each point on the curves represents the average time required for division of 12 individuals. ○, chilomonads from a young culture; ⊙, chilomonads from a medium-aged culture; ●, chilomonads from an old culture. In experiment A, after each division, one of the chilomonads was removed from the culture and discarded. In experiment B, after each division, one of the chilomonads was put into a clean depression containing 0.025 cc fresh acetate-ammonium solution.

Experiment 4.—If X readily diffuses out of the chilomonads, and if the rate of reproduction is closely correlated with the concentration of X in the protoplasm in accord with the postulations made above, one would expect the rate of reproduction to vary inversely with the frequency of transfer to fresh culture fluid.

Two series of observations were made concerning this hypothesis. In one, 24 chilomonads were selected for size and content from a vigorous culture and washed in fresh culture fluid, as described above. One of these was put into each of 24 depressions containing 0.025 cc culture fluid each and transferred to the water-bath. Every half-hour after this the chilomonads in 12 of the depressions were transferred to fresh culture fluid in clean depressions and those in the other 12 depressions were taken up in a pipet and returned to the solution from which they had been taken. The average time required for

the first division was 11 hours for those which had been transferred to fresh culture fluid and only 6.6 for those which had not.

In the second series of observations the methods used were the same as those in the first except that 12 of the chilomonads used were transferred to fresh culture fluid every 15 minutes in place of every 30 minutes. These observations were continued for 15 hours and during that time none of those which had been transferred to fresh culture fluid divided, whereas the average time required for the first division in the twelve which had not been transferred was only 6.25 hours.

The results obtained indicate therefore that the rate of reproduction in *Chilomonas* varies inversely with the frequency of transfer to fresh culture fluid, and they consequently support the conclusion concerning the effect of diffusion of X on the rate of reproduction, reached in the preceding experiments.

DISCUSSION

As previously stated, Robertson concluded that unicellular organisms and probably all other cells produce substances which accelerate growth; Yocom, Petersen, and Dimitrowa obtained results which support this conclusion; and Woodruff, Peskett, Greenleaf, Calkins, Myers, Grimwald, Chejfec, Jahn, Darby, Di Tomo, Beers, Johnson and Phelps obtained results which oppose it, and they conclude that protozoa do not produce growth-promoting substance and imply that the reverse results obtained by Robertson and others are due to differences in the amount or the kind or the availability of the food under the different conditions which obtained in the experiments.

The results presented above demonstrate that the rate of reproduction in *C. paramecium* varies directly with the density of population under some conditions and inversely under others, and that these results are not dependent upon variation in the amount, the kind, or the availability of food but upon the concentration of an unknown substance (X) produced by the chilomonads. However, the fact that such substance is produced by *Chilomonas* obviously does not prove that similar substance is produced by other cells and it therefore does not prove that Robertson's conclusion is valid; but it nevertheless does lend some support to this conclusion. And the facts that Went (1937) and others, as is well known, demonstrated that growth-promoting substance is produced in some plant cells, and that Peebles (1929) and others obtained results which appear to indicate that such substance is produced by eggs, also lend support to it. Moreover, we demonstrated that the rate of reproduction in fresh culture fluid varies with the age of the parent culture. The divergent results obtained by others on the relation between density of population and rate of reproduction may consequently be due—at least in part—to difference in the age of the parent cultures rather than to difference in the food. These results therefore do not necessarily overthrow Robertson's conclusion.

The results presented in the preceding section of this paper seem to prove that *Chilomonas* not only produces growth-promoting substance in accord with Robertson's views but that if this substance is concentrated, it retards and inhibits growth and kills the organisms. If the production of such a substance is widespread among cells, it is obviously of the greatest importance in all problems concerning growth, reproduction, and senescence, and it doubtless has an important bearing on many others.

We are continuing our experiments on *Chilomonas* concerning the nature and the action of the substance X produced by them, and we hope soon to extend them to other forms.

SUMMARY

1. Sterile acetate-ammonium culture fluid and the isolation culture method, with transfer to fresh culture fluid every 24 hours, were used practically exclusively in this work.

2. *Chilomonas* grows indefinitely in mass cultures in acetate-ammonium culture fluid if it is transferred to fresh fluid every other day; but if it is not transferred, it dies out in about 6 days.

3. In isolation cultures transferred to fresh culture fluid every 24 hours the rate of reproduction of *Chilomonas* increases to a maximum as the volume of culture fluid per individual decreases and then decreases to zero.

4. The average number of chilomonads produced in 24 hours at 24 ± 0.2° C., from each specimen introduced, increased in one series of tests from 8.7 with 1 specimen in 0.4 cc culture fluid to 13.65 with 50 specimens in 0.4 cc (more than 50 percent) and in another from 9.15 with 1 specimen in 0.4 cc to 12.98 with 1 specimen in 0.006 cc (over 40 percent).

5. The increase in rate of growth is somewhat greater than the increase in rate of reproduction.

6. If different numbers of chilomonads are left for the same length of time in the same amount of culture fluid, and then removed and chilomonads from a vigorous culture added, the rate of reproduction in the culture fluid increases to a maximum, as the number of chilomonads which had been in it increases, and then decreases to zero.

7. If culture fluid in which *Chilomonas* has died out is subjected to 100° and then cooled, the rate of reproduction of *Chilomonas* in it increases from zero to maximum, as the time at 100° increases, and then decreases to that which obtains in fresh culture fluid.

8. If culture fluid in which *Chilomonas* has died out is diluted with fresh culture fluid, the rate of reproduction of *Chilomonas* in it increases to maximum, as the dilution increases, and then decreases to normal.

9. The relation between the number of chilomonads per given volume of culture fluid and the rate of reproduction is not due to change in the culture fluid, owing to consumption of substance in it or to mitogenetic rays or to contact stimulation. It is correlated with the concentration of a substance produced by the chilomonads.

10. This substance (designated "X") accelerates reproduction in low concentrations and inhibits it and causes death in high concentrations.

11. Fission is not necessary for the production of substance X.

12. The lowest effective concentration of X is produced by one chilomonad in 0.1 cc culture fluid for 4 hours, and the optimum concentration by 75 in 0.1 cc for 4 hours.

13. If 1 chilomonad produces X equal in volume to one-tenth of its volume in 4 hours, the lowest effective concentration is about 1 part X in 800,000,000 parts of culture fluid and the optimum is about 1 part in 10,000,000.

14. If culture fluid in which chilomonads have developed long enough to produce a maximum rate of reproduction (i.e. X in optimum concentration) is subjected to 100° C. for 60 minutes, the rate in it is the same as in fresh culture fluid. If culture fluid in which chilomonads have died out is subjected to 100° C. for 90 minutes, the rate in it is the same as it is in fresh culture fluid.

15. The substance X produced by *Chilomonas* is heat-labile, and the time required to destroy it in a given temperature varies directly with the concentration.

16. The rate of reproduction of chilomonads in culture fluid containing X in a given concentration, varies with the age of the culture from which they were taken. This is due to variation in the concentration of X in the protoplasm, with the age of the culture.

17. If chilomonads in which the concentration of X in the protoplasm is supra-optimum are transferred to fresh culture fluid and then transferred every 24 hours, the rate of reproduction increases to a maximum and then decreases to normal. This is due to decrease in the concentration of X in the protoplasm, owing to diffusion.

18. If chilomonads are frequently changed to fresh culture fluid, the rate of reproduction decreases as the frequency increases. This is due to diffusion and consequent decreases of X in the protoplasm.

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FAT RELEASE IN AMOEBA AFTER IRRADIATION[1]

(One plate)

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IT HAS been shown that ultra-violet radiation and also other stimulating agents cause a release of calcium in protoplasm (Heilbrunn and Daugherty, 1933; Heilbrunn and Mazia, 1936; Mazia and Clark, 1936). This is doubtless not the only effect of such agents. Nadson (1925) found that radium caused a production of fat droplets in the protoplasm of yeast cells and that the same effect was also produced by ultra-violet radiation. In general, it appears that various types of irradiation may release fat or lipoid from protoplasmic combination (for references to literature see Heilbrunn and Mazia, 1936). The evidence on which this statement is based is perhaps not too clear.

By centrifuging cells, it is possible to estimate the amount of free fat in them (Heilbrunn, 1936). In this study we have attempted to discover whether radiation from a mercury arc lamp can cause an increase in the amount of fat which can be centrifuged to one side of a cell.

The work was done on two species of amoeba, *Amoeba proteus* and *A. dubia*. The results were essentially the same for both species, but only data for *A. proteus* are presented. The organisms were exposed for varying lengths of time to the complete radiation from a Uviarc lamp kindly loaned by the General Electric Company. The lamp was run at 100 volts and 3.8 amperes, and the amoebae were placed at a distance of 40–45 cm. from the arc. No attempt was made to determine the intensity of the radiation employed or to isolate particular wave-lengths. The determination of radiation intensity is not simple. Isolation of individual wave-lengths so weakens the intensity that it is difficult to obtain effects on biological material. In our experiments we were not concerned with the relative effect of one wave-length as compared with another, nor were we interested in discovering the intensity necessary to produce an effect. The Uviarc lamp is well known, and its spectrum has been carefully studied (Buttolph, 1930).

In our experiments we sought to use an effective but not lethal intensity of radiation, and we wished to discover whether treatment with such radiation would actually cause a release of fat in amoeba. In experiments of this sort, it is possible to make a rough quantitative measure of the free fat in individual amoebae and to compare the amount in normal and irradiated specimens. If amoebae which normally contain a small amount of free fat are exposed to the radiation from a Uviarc lamp, it can be shown that the amount of the free fat increases following irradiation. However, these experiments are not very satisfactory, for it is difficult to obtain anything better than a rough estimate of the volume of free fat present in an amoeba. It is simpler and more conclusive to conduct experiments on strains or cultures of amoebae which normally are totally lacking in free fat. Such fat-free cultures must be carefully selected, for they are the exception rather

[1] This work has been aided by a grant from the Radiation Committee of the National Research Council.

than the rule. Although we always began with fat-free cultures of amoebae, in some of our experiments, we found it worth while to make conditions favorable to fat release before exposing the amoebae to the Uviarc lamp. This was done by immersing the amoebae for hours or even days in distilled water. Of the amoebae in distilled water, a small percentage showed free fat. This percentage was greatly increased after the irradiation.

TABLE 1

FAT RELEASE AFTER 45 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage of Amoebae Showing Fat Release
June 15	24	7.3	(1)	35−	15–30	11−, 2+	
		7.3	(5)	27−	15–30	5−, 4+	
		.....	(1)	.....	45–60	..... 5+	
		.....	(5)	.....	45–60	..... 11+	
16	24.5	.....	(1)	4−	60–75	3−, 4+	
		.....	(5)	5−	60–75	1−, 3+	
19	26.0	.....	(10)	4−	60	5−	
22	24.0	.....	(8)	4−	45–75	16−, 3+	
24	22.5	.....	(8)	8−	60–75	6−, 5+	
			(10)	11−	75	5−	
25	22.5	.....	(8)	8−	45–60	12−, 3+	
Total	.....	.....	.....	106−	.....	104 (40+)	38.4
Nov. 11	18.5	.....	I	9−	60	4−, 4+	
		7.2	(4)	7−	60	8−	
		7.4	(2)	10−	60	7−	
12	20.0	.....	(2)	28−	60	11−	
30	20.0	6.9	(2)	4−	60	1−, 2+	
		.....	.....	.....	26	10−, 3+	
Dec. 2	20.0	6.9	III	65−	30–45	35−	
		.....	.....	.....	70	12−	
		.....	.....	.....	90–120	35−	
Total	.....	.....	.....	123−(0%)	.....	132 (9+)	6.8

The technique of the experiments was very simple. After exposure to the Uviarc lamp, the amoebae were allowed to remain in suitable dishes for 30–60 minutes. Such a time interval is necessary to allow the process of fat release to take place. At the expiration of the time interval, the amoebae, both irradiated specimens and controls, were centrifuged for 5–10 minutes in an Emerson electric centrifuge at a force approximately six thousand times gravity. The centrifugal treatment was strong enough to stratify the protoplasmic constituents, and the fats of the cell were thrown to the light pole. Here they could readily be stained with Sudan III. This is illustrated in Plate I. In the

upper portion of the irradiated cells, there is an accumulation of dark material, which is colored a brilliant orange in the original preparation. No such material appears in the control.

The tables show three series of experiments, and they give the results of exposures of 45, 60, and 90 seconds. In all the experiments irradiation did cause a very definite release of fat in a fair percentage of exposed individuals. This percentage was always greater when the experiments were conducted in the warmer months. Thus, in Table 1 the June amoebae showed 38.4 per cent with positive results, whereas the November and December amoebae treated in the same way showed only 6.8 per cent.

TABLE 2

FAT RELEASE AFTER 60 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	pH of Distilled Water	Time in Distilled Water	Control Amoebae with (+) or without (−) Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage with Fat Release
Nov. 12...	20	.....	(2)	28−	60	25− ...	.....	.....	....	.....	....	
		.....	(4)	34−	60	21− ...	.....	.....	....	.....	....	
16...	19.5	7.4	(2)	15−	.....	....	6.8	68 hr.	6− ...	76	4−, 2+	
17...	21.5	7.2	(2)	9−	.....	....	.....	2 hr.	7− ...	50	2−, 4+	
21...	20.0	7.5	(4)	15−	.....	....	7.0	21 hr.	7−, 4+	60	6−, 2+	
23...	21.5	7.0	(2)	15−	.....	....	6.5	48 hr.	13− ...	50	.... 4+	
24...	20–22	7.2	(1)	10−	62	13−, ...	.....	.....	....	.....	....	
		7.3	(2)	12−	60	4−, 1+	.....	.....	....	.....	....	
30...	20	6.9	(2)	4−	90	11−, 2+	.....	.....	....	.....	....	
Dec. 2...	20	6.9	III	65−	70	18− ...	.....	.....	....	.....	....	
3...	21	6.7	III	11−	.....	....	6.5	5½ hr.	18− ...	44	.... 7+	
4...	23	6.9	III	25−	58	21− ...	6.5	22 hr.	9− ...	60–65	.... 35+	
		.....	.....	.....	.....	....	.....	.....	....	30–45	.... 19+	
		.....	.....	.....	.....	....	.....	4 hr.	20− ...	60	23−, 2+	
7...	20	7.2	(4)	13−	37	5−, 3+	.....	.....	....	.....	....	
		7.0	III	20−	37	7− ...	6.5	3 days	12− ...	42	5−, 3+	
8...	19–21	7.0	III	.....	.....	....	6.3	24 hr.	6− ...	39	6−, 1+	
		.....	.....	.....	.....	....	.....	.....	....	80	1−, 1+	
		6.9	(2)	.....	125	5−, 2+	.....	.....	....	.....	....	
		.....	(4)	6−	60	16− ...	6.3	24 hr.	7− ...	125	3−, 2+	
		.....	.....	.....	130	7−, 5+	.....	.....	....	.....	....	
Total	.....	.....	.....	282 (0%+)	.....	166 13+= (7.8%+)	.....	.....	109 4+= (3.6%+)	.....	132 82+=	62.1

Tables 2 and 3 give data both for amoebae irradiated directly in culture fluid and for amoebae immersed in distilled water before exposure to the radiation. Following exposure to distilled water, much higher percentages of amoebae with free fat are obtained. The favorable effect of the distilled water is perhaps to be correlated with the fact that such water is lacking in carbonates and bicarbonates. On the other hand, the culture fluid, which consisted essentially of spring water, presumably did contain carbonate or bicarbonate. The presence of such compounds would tend to send carbonic acid into the protoplasm (Jacobs, 1920). Thus, the amoebae in distilled water might have less acid protoplasm than those in culture fluid. It has been shown previously (for the protoplasm of sea-urchin eggs) that acidity tends to prevent fat release (Heilbrunn, 1936). More-

over, in the case of amoeba, we were able to show that treatment with carbon dioxide prevented release of fat by irradiation. On the other hand, if amoeba protoplasm is made more alkaline by immersion in dilute ammonium chloride solutions, then subsequent irradiation causes a greatly increased release of fat and the entire amoeba becomes filled with tiny droplets, presumably of fatty material.

As a whole, the experiments confirm the older observations which have shown that fat release may follow irradiation of protoplasm. Further study is necessary in order to determine whether this fat release is due to a direct action of the radiation on the pro-

TABLE 3

FAT RELEASE AFTER 90 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	pH of Distilled Water	Time in Distilled Water	Control Amoebae with (+) or without (−) Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage with Fat Release
Nov. 13	19	7.4	(2)	.....	90	6−, 1+	.....	.....	....	.....	....	
		7.6	(4)	5−	95	2− ...	.....	.....	....	.....	....	
17	21.5	7.2	(2)	9−	93	4−, 1+	.....	.....	....	.....	....	
18	21.0	7.7	(4)	4−	60	.... 3+	7.0	22 hr.	2−, 3+	30	.... 5+	
21	20.0	7.2	(2)	20−	.....	....	7.0	21 hr.	16−, ...	75	.... 9+	
		7.5	(4)	15−	60	10− ...	7.0	21 hr.	7−, 4+	75	1−, 2+	
23	21.5	7.0	(2)	15−	30–60	23− ...	6.5	48 hr.	13− ...	30–60	10−	
24	20–22	7.0	(2)	12−	70	19− ...	6.5	19 hr.	13− ...	60	.... 10+	
		7.3	(4)	12−	70	7−, 2+	.....	.....	....	.....	....	
30	20.0	6.9	(2)	4−	75	8− ...	6.5	5 days	9− ...	28	11−, 3+	
		7.2	(4)	5−	75	12−, 3+	.....	.....	....	.....	....	
Dec. 2	20.0	6.9	III	65−	70	10− ...	.....	.....	....	.....	....	
3	21.0	6.9	(4)	13−	120	6− ...	.....	.....	....	.....	....	
4	19–21	7.1	(4)	6−	90	5−, 1+	.....	.....	....	.....	....	
10	21.0	.....	III	12−	.....	....	.....	43 hr.	8−, 1+	68	3−, 5+	
Total	.....	.....	.....	197 (0%+)	.....	123 11+= (8.9%+)	.....	.....	76 8+= (10%+)	.....	59 34+=	57.6

tein-lipid binding or whether it may not be due indirectly to an alkalinization of the protoplasm. For it is conceivable that irradiation makes the protoplasm more alkaline and that this alkalinization produces a release of bound fat.

Irradiation is one type of stimulation. It seems not unlikely that other types of stimulation may also cause a release of fat in the protoplasm. This question offers possibilities for further research, and such work is at present being attempted in this laboratory. Preliminary results indicate that protoplasmic protein may be combined with both fat and calcium, and that both fat and calcium may be released in stimulation. Those types of stimulation which may involve localized increases in temperature seem especially prone to release fat.

SUMMARY

Radiation from a mercury arc lamp causes a release of fat in amoeba protoplasm.

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PLATE I

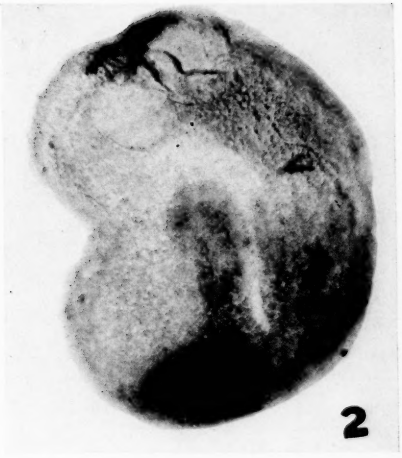
FIG. 1.—*Amoeba proteus*, centrifuged control. ×300.

FIG. 2.—*Amoeba proteus*, centrifuged after 45 seconds of irradiation. Time interval between irradiation and centrifugal treatment, 60 minutes. ×385.

FIG. 3.—*Amoeba dubia*, centrifuged control. ×290.

FIG. 4.—*Amoeba dubia*, centrifuged after 45 seconds of irradiation. Time interval between irradiation and centrifugal treatment, 75 minutes. ×280.

PLATE I



UNIV. OF MICH.

AN EXPERIMENTAL STUDY OF CLOT FORMATION IN THE PERIVISCERAL FLUID OF ARBACIA[1]

(One plate)

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IT IS well known that, if the body fluid of a sea urchin is withdrawn from the shell, it undergoes a change in which the cells separate completely from the fluid to form a clot. When the membrane or shell is injured in any way, the body fluid will clot at the point of injury and close in the opening (Kindred, 1921). Similar injury to the intestinal wall by sand or sharply pointed intestinal contents will also result in clot formation.

These observations suggested an investigation to determine, if possible, just why the body fluid should clot under these circumstances and not under normal conditions within the shell. In attempting to solve this problem, it was thought advisable to test the effect on the body fluid of different reagents, such as tissue extracts, fat solvents, salt solutions, or other reagents that might be suspected of being able to influence clot formation either by preventing or by causing it.

Previous investigators in this field have dealt more with the morphology of the clot than with the physiological problem suggested by its formation. Cuénot (1891) distinguishes two different series of cells from which he believes all the cellular elements are derived. The first series, which he calls "vibratile globules," are minute spherical elements with a head and vibratile tail. These cells rotate very rapidly and continuously, and their function, according to Cuénot, is to keep the fluid contents of the body cavity in motion.

The second series were called "amibocytes" and were subdivided into several different types. The cells of the first type of this series are the colorless "amibocytes" having a homogeneous cytoplasm and numerous filiform pseudopodia which sometimes unite to form complete rings. The name "leucocyte" was applied to these cells by Goodrich (1919), and many investigators believe that they are the chief agents in the formation of the clot. Further details concerning the morphology of clotting may be found in papers by Kindred (1921) and Theel (1896).

Geddes (1880) refers the clot formation to the remarkable massing-together of the leucocytes to form a network in which the other corpuscles become entangled. Kindred also observed this phenomenon but failed to determine whether these massed cells formed true plasmodia, as Geddes contends, or retained their individuality as later writers hold. E. A. Schäfer (1883) maintains that clotting is due to the exuding of what he calls "coagulable material" by the leucocytes when the body fluid is removed from the shell. This "coagulable material" is unlike the fibrin of vertebrate blood but appears

[1] A thesis in zoölogy presented to the faculty of the graduate school of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

to be closely allied to mucin. Schäfer's paper, however, was only a preliminary notice, and it seems that he never completed his investigation.[2]

METHODS OF STUDY

The methods used in these experiments are comparatively simple. The procedure followed was to determine the difference in time of clotting of normal body fluid and of body fluid after treatment with different reagents. Equal amounts (8 cc.) of the body fluid from the same animal were placed in two different paraffined Stender dishes. Two cc. of the tissue extract or of the reagent to be tested were then added to the fluid in one container and an equal amount of sea water added, as a control, to the fluid in the second container. The difference in time of clotting was then observed. Clotting was determined to have taken place when the corpuscles were precipitated in typical clot formation. This precipitation is made more distinct by the accompanying pigment liberation. The end point is reasonably sharp and can scarcely be mistaken. Whenever necessary, clotting could be hastened by rotating the container. It was found to be practically impossible to remove the body fluid without its showing some signs of clotting. In order to prevent clotting from taking place too rapidly, it was found necessary to withdraw the fluid from the animal by means of a finely pointed, well-paraffined pipette. The point of this pipette had been rounded by heating in order to decrease the amount of injury to the membrane when the pipette was injected through it.

Another method was to inject 1 or 2 cc. of the reagent into the body cavity after first making a small opening in the membrane opposite to the point of injection in order to allow the escape of excess fluid. The membrane was then carefully removed to determine if the injected material had caused clotting within the shell.

RESULTS

Cellular nature of clot.—As already pointed out, certain observers had noted the cellular nature of the clot. It was thought possible, however, that in addition there might be some intercellular clotting. To determine if any such clotting took place, it was necessary to separate the cells from the fluid. This was accomplished by filtering the body fluid as it flowed from the shell. All the cells remained on the filter, and a clear fluid passed through. This fluid gave no coagulation on dilution; but, if the cells were suspended in water, they did form a typical clot. There was no plasma clot formation as is characteristic of certain other invertebrate forms.

Action of tissue extracts.—The well-known influence of tissue extract on vertebrate blood suggested testing the effect of extracts of invertebrate tissues on the body fluid of the sea urchin. These extracts were prepared by thoroughly grinding the tissues in the mortar crucible and then filtering with a suction filter.

A tissue extract of the shell of *Arbacia* minus the spines and all the internal structures causes clot formation within a few seconds. Extracts of the intestine or of any of the other internal structures will produce the same effect. These extracts are effective in dilutions of one to fifty.

Extracts of starfish, crustaceans, and mollusks gave the same results as *Arbacia* extracts. The crustacean extracts were muscle extracts, whereas extracts of the entire

[2] This problem was suggested by Dr. L. V. Heilbrunn. I wish to take this opportunity to express my appreciation for his kind advice and criticism.

animal were used in other cases. Some of these extracts such as those of *Limulus*, the blue crab, *Venus mercenaria*, and *Mactra* were even more effective than *Arbacia* extracts. Extracts of frog's muscle had no effect on clot formation. Thrombin and cephalin likewise had no effect. The fact that vertebrate muscle extracts have no effect on invertebrate clotting has been reported by Nolf (1909).

All these extracts also brought about a liberation of the pigment of the cells, changing the color of the body fluid from red to orange.

An extract of previously clotted material seems to hasten clotting with the same effectiveness as extracts of *Arbacia* and other invertebrates. This extract was prepared by grinding a large amount of clotted material in a mortar crucible in which ground glass had been placed.

TABLE 1

Extracts	Average Time of Clotting (Seconds)	Control (Seconds)
Arbacia (shell)	52	3,318
Starfish	70	5,200
Limulus	28	4,098
Blue crab	34	3,024
Spider crab	49	3,150
Venus mercenaria	40	3,780
Mactra	38	2,760
Ostrea	58	4,356
Shrimp	83	5,130
Boiled *Arbacia* eggs	58	2,988
Broken *Arbacia* eggs	68	3,360
Clotted material	42	3,940
Sperm	96	4,632

Arbacia eggs which have been broken up in ground glass, or eggs which have been boiled, will also cause clot formation. Sperm extract has the same effect. Whole eggs which have not been boiled seem to have no effect on clot formation.

At least twenty-five experiments were performed with each extract. Concentrated extracts were used in all cases. The average time of clotting in these experiments is listed in Table 1.

The rather high average time of clotting in the controls in the case of shrimp, starfish, and sperm is probably due to the fact that in these cases the body fluid of fresh *Arbacia* was used. The body fluid of fresh *Arbacia* seems to clot more slowly than that of *Arbacia* kept in an aquarium for four or five days.

These extracts were also injected into the body cavity, and, when the membrane was removed, the fluid had already clotted within the shell. Such clotting does not take place under normal conditions when the membrane is removed very carefully (Pl. I, Figs. 1 and 2).

Certain investigators have described a breakdown of what they call pigment granules imbedded in the jelly of the sand dollar egg when the eggs are broken under pressure. They have also noticed the fusion of these granules at different times. These so-called pigment granules resemble very closely the pigmented cells found in the body fluid of the sand dollar. It seems probable that in reality they are pigmented blood cells that have

been caught in the jelly of the egg. The pigment granules of these cells are dispersed throughout the cells in the same manner as they are in the body fluid cells. There also seems to be present a definite nucleus and cell membrane which become even more definite after the pigment granules have broken down. These same cells can be found at various places in the ovaries and testes and are not confined to the egg jelly. When the egg is broken under pressure and the exudate comes in contact with these cells, the pigment granules break down just as the pigment granules of the body fluid cells of *Arbacia* break down when treated with *Arbacia* egg extracts.

Action of K and Ca.—It is well known that the inorganic ions play an important role in physiological processes, and for this reason it was thought advisable to test the effect of some of these ions on the body fluid of the sea urchin.

An isotonic solution of KCl or of any of the K salts appears to have exactly the same effect as the tissue extracts. If 2 cc. of this solution is added to 8 cc. of body fluid, a clot

TABLE 2

Salt	Average Time of Clotting (Seconds)	Control (Seconds)
0.3 M K_2SO_4	96	3,689
0.53 M KCl	82	3,258
0.53 M KNO_3	68	4,268
0.53 M KSCN	42	3,962
0.3 M $CaCl_2$	324	3,810
0.3 M $BaCl_2$	402	4,319
0.3 M $SrCl_2$	385	3,728

appears within a few seconds and pigment liberation also takes place. This isotonic solution can be diluted twenty times and still be effective (Fig. 3).

$CaCl_2$ also hastens clotting but not to the same extent as KCl. $BaCl_2$ and $SrCl_2$ appear to have the same effect as $CaCl_2$.

Solutions of 0.53 M KCl and 0.3 M $CaCl_2$ were used as isotonic solutions, although some authors give slightly higher molarity values. However, 0.53 M solutions in the case of monovalent ions and 0.3 M in the case of divalent ions are commonly used as isotonic solutions because in such solutions *Arbacia* eggs neither shrink nor swell.

The K salts appear to be effective in the following order: $SO_4 < Cl < NO_3 < SCN$ (see Table 2). If these solutions are injected into the body cavity, clotting will take place within the shell.

The white or colorless amoeboid cells in the body fluid seem to be composed of granules of the same size and shape as those in the red amoebocytes, and these white granules also appear to break down under the influence of K and Ca.

Action of fat solvents.—Solutions of fat solvents appear to have an accelerating effect on clot formation. These solutions were as effective as the KCl solutions and also brought about pigment liberations. Comparative results are shown in Table 3.

Temperature effects.—Gradual exposure of *Arbacia* to temperatures ranging from 0° C. to 10° C. seems to have a slightly inhibitory effect on clot formation. However, if the body fluid is suddenly placed in a Stender dish which has been cooled to 0° C., the pigment granules break down immediately, and the cells form a clot.

In the same way gradual exposure to high temperatures has no observable effect unless the *Arbacia* is killed by heating. At temperatures above 40° C. the body fluid seems to be permanently altered and will not clot. However, if the body fluid is suddenly placed in a Stender dish which has been heated to a temperature of 40° C., pigment liberation and clotting ensue within a few seconds.

Effect of foreign bodies.—If a piece of boiled intestine or a piece of the membrane or a grain of sand is introduced into the body fluid, the blood cells accumulate around it and, by agglutinating, form a solid mass somewhat similar in appearance to the formation of a thrombus around a foreign body introduced into the blood vessel of a vertebrate. In the case of the sea urchin this thrombus-like mass consists of clotted cells. The cells react in the same way to any foreign body. If any air bubbles are in the fluid, a clot can be seen to form around the outer edges.

TABLE 3

Fat Solvent	Average Time of Clotting (Seconds)	Control (Seconds)
2 per cent ether	56	4,260
5 per cent acetone	66	3,696
0.13 per cent chloroform	48	3,228
2.5 per cent chloral hydrate	72	4,020
2 per cent acetonitrile	42	4,158
5 per cent alcohol	82	2,970

Mechanical stimulation.—If we remove the body fluid from the body cavity with extreme care and then place a drop of this fluid on a slide and examine it microscopically, a marked difference can be noticed between the cells suspended in the plasma and those that have dropped to the surface of the slide. In the former almost all the granules remain unchanged, and no clotting of the cells has taken place. In the latter the cell granules are broken down to a great extent, and clotting has taken place. The mechanical irritation of the slide appears to produce pigment liberation and clotting. If the fluid is stirred with a glass rod, the cells readily adhere to it; and, upon examination, it is found that the pigment granules of such cells have broken down. If some of the cells are placed on a slide under a cover slip, and the water removed from under the cover slip until it exerts a pressure on the cells, the pigment granules can be seen to break down immediately, and cells that are close to one another seem to fuse to form small individual clots (Figs. 4 and 5).

Electrical stimulation.—Passing a direct current through the body fluid appears to hasten clotting considerably and also to bring about pigment liberation. Clotting takes place under these circumstances in about 4 or 5 minutes while the controls clotted in an average time of 35 minutes.

The electrodes were prepared in the following manner: glass tubes of 9-mm. bore were filled at their tips with a 5 per cent agar gel made up in tap water. A saturated solution of $CuSO_4$ was placed over the gel, and the copper wires dipped into this. The body fluid was placed in a 2-inch Stender dish and the electrodes placed at both extremes of the dish. A current of 5 milliamperes was allowed to flow through the fluid until pigment liberation and clotting took place.

Irradiation with ultra-violet light.—The body fluid was irradiated from a Cooper-Hewitt quartz mercury vapor lamp, at a distance from the arc of 28 centimeters. The fluid was exposed for different intervals of time and then examined. Pigment liberation and clotting took place after the cells had been exposed for 4.5 minutes, the controls in this case clotting in an average time of 42 minutes. Longer exposure produced a more profuse liberation of pigment. A single typical clot is not formed under these circumstances, but the cells seem to adhere to one another so as to form a number of small isolated clots.

Effect of hypertonic solutions.—Hypertonic sea water also hastens clotting and brings about pigment liberation. These hypertonic solutions were made by adding 8 cc. of 2.5 M NaCl solution to 50 cc. of sea water. Under the influence of these solutions pigment liberation and clotting took place in about 3 or 4 minutes while the controls clotted on an average of 39 minutes.

Anticlotting agents.—The reaction of the body fluid cells to stimulating agents suggested that perhaps clotting may be prevented by anesthetic agents such as Mg, although the fat solvents reacted in just the opposite manner and hastened clotting. Reagents such as oxalate and citrate which act as anticoagulants in vertebrate blood clotting were also tested.

If an equal amount of a saturated solution of $MgSO_4$ is injected into the body cavity or added to the body fluid as it flows from the shell, clotting will be indefinitely delayed. An isotonic solution of $MgCl_2$ will also inhibit clotting if added in the proportion of about three parts of $MgCl_2$ to one part of body fluid.

If an equal amount of isotonic $MgCl_2$ is added to an isotonic solution of KCl or $CaCl_2$, the K and Ca seem to be antagonized by the Mg and do not cause clotting or pigment liberation. The same is true if an equal amount of isotonic $MgCl_2$ is added to the tissue extract; the tissue extract also seems to be inhibited and does not cause clotting or pigment liberation.

A 3 per cent solution of formalin will also inhibit clot formation. In this case, however, the cells are evidently killed as they move about very feebly or do not move at all. The cells seem to react to this solution in the same manner as when they are heated to high temperatures, i.e., they assume a spherical shape and withdraw their pseudopodia.

A 10 per cent solution of peptone will also inhibit clotting.

Sea water with a pH between 4.2 and 4.6 has a marked inhibitory effect on clot formation but does not prevent it entirely. There are no gross signs of typical clot formation, but small clumps of cells may be observed under the microscope.

If the body fluid is mixed while flowing from the shell with about twice its volume of 0.3 M K-oxalate at pH 7.8, pigment liberation does not take place and clotting is indefinitely delayed. The fluid must be transferred here from the shell to the oxalate very rapidly and must be mixed thoroughly by stirring. The cells not only do not agglutinate to one another but do not adhere to foreign bodies.

Pigment liberation and clotting can also be inhibited by the same process in 0.27 M Na citrate at pH 7.8. In both the oxalate and citrate solutions the cells, both pigmented and nonpigmented, take on a rounded appearance and withdraw their pseudopods.

When body fluid is added as a control to sea water of the same volume as the oxalate and citrate, clotting takes place normally.

If tissue extracts, K salts, or hypertonic solutions are added to oxalated or citrated body fluid, neither pigment liberation nor clotting takes place. Mechanical stimulation, ultra-violet irradiation, or an electric current also have no effect upon material so treated.

The fat solvents, however, do bring about pigment liberation in both the oxalated and the citrated material. Perhaps these reagents are able to penetrate somewhat more deeply into the interior of the cell and cause the ionization of Ca which may be bound there.

DISCUSSION

As a result of these experiments it would appear that normal clotting in the body fluid of *Arbacia* is induced by the liberation of an injury substance when any tissue of the body is damaged. This seems to be evident from the effect of tissue extracts on clotting and from the fact that, if a needle is pushed through the membrane and the inner wall of the test scratched, a clot appears in that region. The effect of this injury substance can be duplicated by K and Ca and certain other reagents. That this substance is not K is evident from the fact that the tissue extract is effective in much higher dilutions than is K. However, from the action of K and Ca on the body fluid, one would be led to venture a suggestion that perhaps both these ions indirectly produce the injury substance.

In some respects clotting of the body fluid of the sea urchin is related to stimulation, in others to clotting as it occurs in vertebrates. Whenever the cells of the body fluid are stimulated either by chemical or by physical means, a breakdown of pigment granules results. Clotting of the cells is always accompanied by this granule breakdown, and any reagent which prevents pigment liberation will also prevent clotting. Leo Loeb (1905), working upon clotted body fluid of *Limulus*, noticed this association and suggested that "it might be very well possible that the same conditions which change the shape and produce the stickiness of cells have also a dissolving influence on the granules." It is also very probable that the breakdown of pigment granules is concomitant with the release of calcium from the surface of the cells as this reaction can be prevented by oxalating or citrating the body fluid. Tissue extract and certain chemical and physical stimulants are evidently able to bring about this release of Ca from the surface of the cell but are prevented from so doing by the presence of oxalate or citrate. All these results appear to be in conformity with the hypothesis developed by Heilbrunn, that a primary effect of stimulation is the release of calcium from combinations located in the periphery of the cell. This release of Ca in the case of the cells of the body fluid seems to be intimately related to clot formation.

It is also interesting to compare the release of pigment from the cells of the body fluid when tissue extracts and KCl are added, with the loss of pigment in *Arbacia* eggs following cytolysis or injury. It is not improbable that there is more than a surface similarity between these two phenomena.

A like phenomenon is also present in the case of the yellow pigment granules in *Arenicola* larvae. The experiments of Lillie (1909) were repeated, and it was found that the same reagents that cause pigment liberation in cells of the body fluid of *Arbacia* also produce pigment liberation in *Arenicola* larvae. After the pigment granules have been broken down, the larvae agglutinate to one another, forming fairly large masses in somewhat the same manner as the body fluid cells agglutinate. Ralph Lillie first described this phenomenon but attributed the release of pigment to an increase in permeability after treatment with certain salts such as chlorides of K, Ca, and Na. This view hardly seems in agreement with the belief that Ca decreases permeability. This release of pigment can also be prevented by oxalating or citrating the larvae. This reaction, therefore, seems to be dependent upon the presence of Ca.

The problem presented here is similar in some respects to the problem upon which so much experimental work has been done in the case of the higher vertebrates. For here

too we have a circulatory medium which does not clot while in the animal's body but coagulates whenever the body wall is wounded and the body fluid is in danger of being lost. It seems strange that so little experimental work has been done on this problem in the case of the invertebrates. Evidence derived from more experimental work on the lower forms may very probably throw some light on the amazingly large number of contradictory theories presented as an explanation of blood-clotting in the higher vertebrates. Certainly, there is some similarity between the two forms of clotting. We know that for clotting to take place in the higher forms at least three things are necessary: Ca, the tissue factor, and thrombin under the influence of which fibrinogen is converted into fibrin. In the case of the sea urchin it seems probable that Ca and the tissue factor must also be present before clotting can take place. It seems probable, also, that something necessary for clotting may be contributed by the cells. As long as the cells are intact, the body fluid does not clot; but, as soon as the pigment granules break down, clotting takes place. It seems evident that something is liberated from the cells with the breakdown of pigment granules that has effect on clotting. The fact that some substance can be extracted from previously clotted material which will cause clotting in freshly shed body fluid also lends support to this belief. This substance contributed by the cells may be somewhat of the nature of an agglutinin which is present in the cells in an inactive state and is activated by the tissue factor just as thrombin may be activated by the tissue factor in mammalian blood. This agglutinin-like substance may be the "coagulable material" which Schäfer (1883) believes to be responsible for clotting in the sea urchin. Just how these three factors—Ca, the tissue factor, and the agglutinin-like substance—react to cause clotting is somewhat a matter of speculation just as it is a matter of speculation as to how Ca, the tissue factor, and thrombin react to cause clotting in mammalian blood. This problem can be solved only by further experimentation.

More experimental work in the different animal phyla and a comparison of the conditions existing in them may throw some light on the stages through which this property of clotting, so highly exhibited in mammalian blood, was developed in the process of evolution.

SUMMARY

1. The clot is due to cellular elements, and no clotting can occur after these are filtered off.

2. *Arbacia* extracts bring about clot formation when added to the body fluid. Extracts of crustaceans, mollusks, and starfish produce the same effect, some being even more effective than *Arbacia* extracts. *Arbacia* eggs broken up in ground glass or boiled also hasten clot formation. Sperm extract has the same effect. These extracts also bring about a breakdown of the pigment granules.

3. K, Ca, Ba, and Sr also cause clot formation and granule breakdown, K being most effective.

4. Dilute solutions of fat solvents bring about clotting in the body fluid.

5. The addition of a foreign body to the perivisceral fluid will cause a clot to form around it.

6. Hypertonic solutions, electrical stimulation, ultra-violet irradiation, and mechanical stimulation also cause pigment liberation and clotting.

7. $MgCl_2$ prevents clotting and also inhibits the action of any of the agents causing clotting. A 10 per cent solution of peptone will also inhibit clotting.

8. Sea water with a pH between 4.2 and 4.6 has a marked inhibitory effect on clot formation.

9. If the body fluid is thoroughly oxalated or citrated, neither pigment liberation nor clotting takes place regardless of the type of stimulus.

10. Clotting in the perivisceral fluid is normally induced by the liberation of an injury substance when any tissue of the body is damaged.

11. Clotting in the body fluid of *Arbacia* is related in some respects to stimulation, in others to vertebrate blood clottings.

12. The results seem to be in agreement with the hypothesis that, when stimulants act on cells, they cause a release of calcium from combinations located in the periphery of the cell. In the case of the cells of the body fluid, this release seems to be intimately associated with clot formation.

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PLATE I

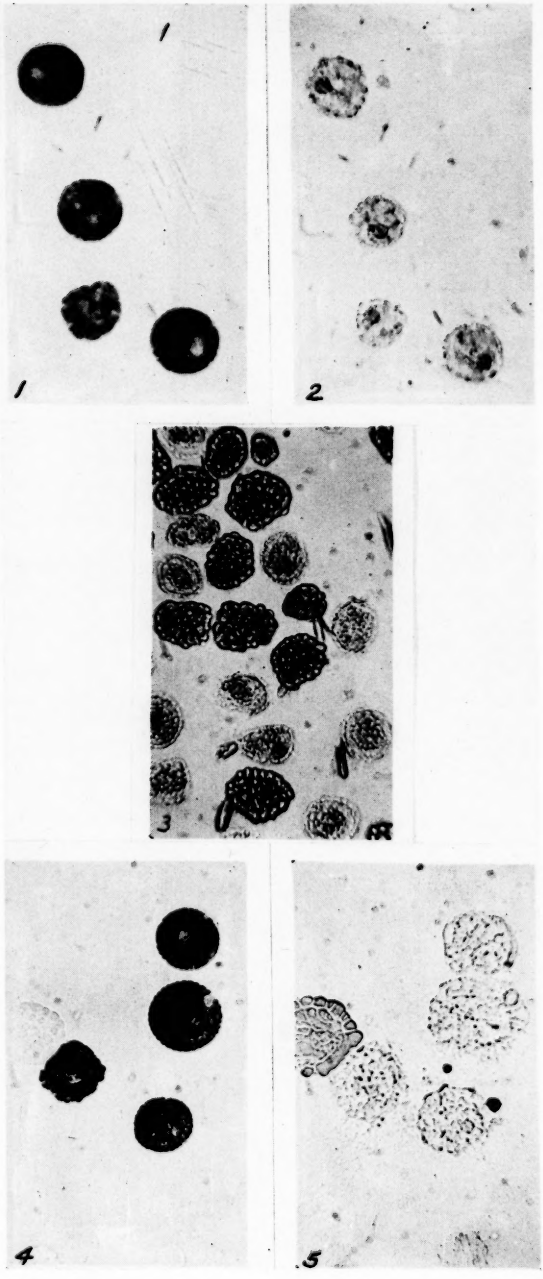
FIG. 1.—Cells before granular breakdown.

FIG. 2.—The same cells in which the granules have broken down after treatment with *Arbacia* shell extract.

FIG. 3.—Cells undergoing granular breakdown after treatment with KCl. In some of the cells the granules have already broken down, in others they are just about to break down.

FIGS. 4 and 5.—Cells before and after granule breakdown under pressure exerted by the cover slip when water was removed from under it.

PLATE I



UNIV. OF MICH.

ABUNDANT FEEDING FOLLOWED BY RESTRICTED FEEDING AND LONGEVITY IN DAPHNIA[1]

(Four figures)

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VARIOUS studies have been made to determine the effects of underfeeding different animals during the early part of life, followed by abundant feeding for the remainder of life. Studies by Jackson and Stewart (1920) and by Osborne and Mendel (1915) with the albino rat showed that early suppression of growth by limitation in food supply did not cause a loss of ability to resume growth when the diet was made adequate at a later time. A similar study by McCay, Crowell, and Maynard (1935) gave similar results and showed, in addition, that the life-span of male rats was increased when they were given a limited amount of food for a considerable period early in life and were well fed later.

Of chief importance for the study to be reported in this paper is the work of Ingle (1933) and of Ingle, Wood, and Banta (1937), who, using cladoceran material, showed that limitation in amount of food early in life, followed by good feeding for the remainder of life, was an effective method of prolonging the life-span in *Daphnia longispina*. The present paper is concerned with the effect to be obtained by subjecting *D. longispina* to limitation in amount of food at a later period in life after the animals had been well fed during an earlier period.

Cladoceran material is suitable for such experimental work because consideration of hereditary characteristics affecting growth, longevity, and reproduction may be disregarded in view of the fact that *D. longispina* reproduces by diploid parthenogenesis.

MATERIALS AND METHODS

One clone of *D. longispina* was used for all the experiments to be reported here. The animals were taken from Dr. Banta's Line 1284, a line which has been maintained in the laboratory by parthenogenetic reproduction since it was started from animals taken from the wild at Ocala, Florida. All the experimental animals were parthenogenetically produced females. This line is maintained in the laboratory in Banta's (1921) manure infusion medium, and this medium or a dilution of it was used throughout these experiments.

Under ordinary laboratory conditions parthenogenesis is normal, and sexual reproduction occurs only when it is purposely initiated. A young female at birth measures about 0.6 mm. in length, is free swimming, and resembles the adult female except in size and form of brood chamber and condition of ovaries. Under normal conditions (unlimited food and room temperature) the young animal passes through the three juvenile instars in about that number of days. The fourth instar, during which eggs appear in the ovaries, is the adolescent instar. At the beginning of the fifth instar, the

[1] The author is indebted to Dr. A. M. Banta for many thoughtful suggestions and much helpful criticism during the course of this work.

first adult instar, the first clutch of eggs passes from the ovaries into the brood chamber, where they are retained during development. At the close of this instar, young are released from the brood chamber and a generation cycle is completed. If culture conditions are favorable, an individual mother may live 18–20 or more instars, produce 200–300 or more young, and attain a maximum size of about 2.8–3.0 mm.

Since the present experiments were concerned with longevity, it was a matter of prime importance to start an experiment with young animals of known age; and it was considered advisable, though not imperative, that all animals used in an experiment should be of the same age. In every series except one, all the animals used had been released within a period of 6 hours. To obtain a large number of animals of the same age, about 150 mothers were taken from the laboratory stock and put separately into 200-cc. wide-mouthed bottles each containing about 100 cc. of manure infusion medium. These stock mothers were kept in an incubator at 25° C. and were transferred to fresh bottles every time they released a brood. The fourth, fifth, or sixth broods produced by these mothers were taken to start a series; the size of such broods was generally over 30, and at the time of their release the mothers appeared to be in prime condition.

In order that all groups of a series be comprised of comparable animals, so far as possible, the young of each brood were distributed uniformly among the various experimental and control groups. Two control groups were provided in each series. One, the normal controls, consisted of mothers cultured throughout life in the normal culture medium containing an abundance of food; the other controls ("semistarved controls") were a group cultured in a medium prepared by diluting this normal medium some thirty to forty times with pond water. Such dilute medium provided a relatively limited amount of food, which permitted animals to grow and reproduce only at a much lower rate than did mothers of the normal control group. Various experimental groups were provided by maintaining a number of animals in normal medium for a definite number of instars and then introducing them into dilute medium at the beginning of a definite instar. At the beginning of a series each animal was placed in a separate 200-cc. wide-mouthed bottle containing about 100 cc. of medium. The bottles were placed in trays and kept in an incubator maintained at a temperature of 23°–26° C. Because there was a difference of about 1° between the top and the bottom of the incubator, it was found advisable to reverse the position of the trays within the incubator from time to time.

Each animal was examined every 12 hours, and such animals as had produced a brood and molted since the preceding observation were measured with a microscope equipped with a micrometer eyepiece before being transferred to a bottle containing a fresh supply of the appropriate medium. The young produced were counted and discarded. All deaths of experimental animals were recorded at these 12-hour intervals.

RESULTS

The results of four complete series of experiments are reported here; they are referred to as Series I, II, III, and IV, according to the chronological order in which they were conducted.

EFFECT ON LONGEVITY

Series I was composed of four different groups, as listed in Table 1, which shows the average longevity for the animals in each group, together with average reproductive life, average number of young per mother, etc. The well-fed controls (normals) had an

average life-span of 30.3 days, a figure exceeded by the semistarved ("retarded") controls by about 27 per cent (38.6 days). The normal controls produced, on the average, more than three times as many young as did the retarded controls, and the reproductive index (average number of young per mother per day of reproductive life) was nearly five times as high for normal as for retarded controls. Table 1 also shows that the average longevity for animals well fed to the sixth instar (29.1 days) was only slightly different from the average (30.3 days) for normal controls. But the animals well fed to the beginning of the twelfth instar and then given the same limited food as the retarded controls had a considerably shortened average life-span (25.8 days), as compared with normal controls, the difference (4.5 days) for these two groups being about 15 per cent of the average for normals.

Survival curves (Fig. 1)[2] showing the percentage of each group surviving each day in Series I throw light on certain points not apparent in Table 1. An examination of these curves reveals that animals in the semistarved control group were more variable with respect to longevity than were animals in the normal control group. The same was true for the group well fed to the sixth instar (about the sixth day), as compared with normal animals; but less variability was observed in the group well fed to the twelfth instar (about the eighteenth day). In other words, the slopes of the curves for normals and for animals well fed to the twelfth instar are relatively quite steep, showing that animals in these groups died off at a considerably faster rate than did animals in the other two groups.

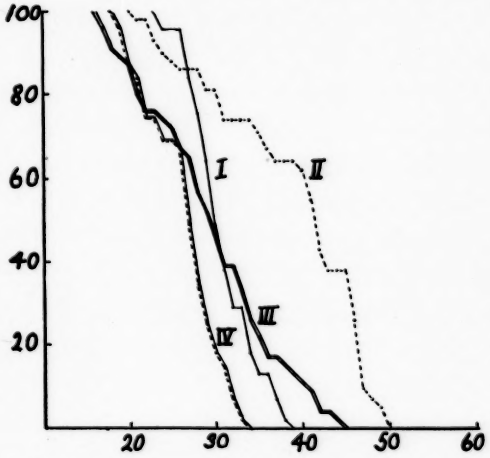

FIG. 1.—Percentage of each group surviving each day, Series I. Ordinates, percentage surviving; abscissas, days of survival. *I* represents well-fed controls; *II*, semistarved controls; *III*, group well fed to sixth instar; *IV*, well fed to twelfth instar.

Table 1 also presents the data for Series II, which was similar in design to Series I except for the inclusion of two additional test groups. The results obtained in Series I had suggested that, if the limited food supply were imposed somewhat earlier than the sixth instar upon previously well-fed animals, an average prolongation of life might be effected. To test this, a group well fed to the beginning of the third instar was included in Series II. Likewise, a group well fed to the beginning of the ninth instar was included, to determine whether at this stage the animals might respond to limited food in about such a manner as did a group well fed to the sixth instar or whether such a group might react to the indicated change more after the fashion of animals deprived of an abundance of food at the twelfth instar. Series II was clearly a long-lived series as compared with Series I.

[2] In the accompanying figures retarded controls are the same as semistarved controls.

TABLE 1

DATA ON LONGEVITY AND REPRODUCTION

Group	No. of Mothers	Longevity (Days)	Reproductive Life (Days)	Young per Mother	Reproductive Index	No. of Broods	Young per Brood
				Series I			
Normals	45	30.30	24.16	269.40	11.30	11.63	23.11
Semistarved	42	38.62	32.10	74.50	2.40	13.10	5.76
Well fed to sixth instar	46	29.08	23.38	99.46	4.25	10.15	9.80
Well fed to twelfth instar	57	25.77	19.98	165.53	8.28	9.44	17.54
				Series II			
Normals	47	36.61	29.66	260.62	8.79	11.47	22.73
Semistarved	34	46.35	36.63	78.44	2.14	14.15	5.54
Well fed to third instar	39	40.52	32.85	69.67	2.12	13.49	5.17
Well fed to sixth instar	38	39.12	32.33	71.21	2.20	12.84	5.54
Well fed to ninth instar	46	28.95	22.10	111.78	5.06	9.22	12.13
Well fed to twelfth instar	51	28.08	20.78	162.04	7.80	8.82	18.36
				Series III			
Normals surviving ninth instar	43	33.49	26.62	213.44	8.02	9.98	21.39
Semistarved surviving ninth instar	35	37.16	30.00	99.43	3.31	11.26	8.83
Well fed to ninth instar surviving ninth	33	26.03	19.56	73.30	3.75	5.48	13.36
Graduated ninth to twelfth instar surviving ninth	33	24.32	17.77	66.61	3.75	4.36	15.26
Normals surviving twelfth instar	41	34.30	27.40	219.37	8.01	10.24	21.41
Semistarved surviving twelfth instar	34	37.69	30.54	101.03	3.31	11.44	8.83
Well fed to twelfth instar surviving twelfth	40	28.76	22.08	135.30	6.13	6.95	19.47
				Series IV			
Normals surviving ninth instar	48	33.49	26.67	161.42	6.05	9.38	17.22
Semistarved surviving ninth instar	48	36.86	28.69	81.96	2.86	11.17	7.34
Well fed to sixth instar surviving ninth	46	32.07	24.25	98.98	4.08	9.20	10.76
Well fed to ninth instar surviving ninth	48	31.23	24.30	126.25	5.20	9.31	13.56
Graduated ninth to twelfth instar surviving twelfth	39	30.60	23.96	134.26	5.60	9.31	14.42
Normals surviving twelfth instar	40	36.30	29.58	178.33	6.03	10.35	17.23
Semistarved surviving twelfth instar	45	37.90	29.60	84.31	2.85	11.51	7.32
Well fed to twelfth instar surviving twelfth	32	30.33	23.64	118.00	4.99	7.66	15.41

In Series II the semistarved control animals lived 25 per cent longer than normal controls. In this experiment the groups well fed to the third and to the sixth instars gave very similar results and showed a fair increase in longevity over the normal animals. They did not, however, approach the semistarved control group in longevity. The data (Table 1, Ser. II) for the groups well fed to the ninth and twelfth instars are in rather close agreement, especially as regards average longevity. The results obtained here seem to indicate that between the sixth and the ninth instars well-fed *Daphnia*, become physiologically incapable of passing from an environment rich in food into one containing only a limited food supply without suffering a resultant decrease in longevity, whereas until or before the sixth instar they seem physiologically capable of becoming adjusted to the change to the extent that the average longevities of the groups may be increased as compared with normal controls.

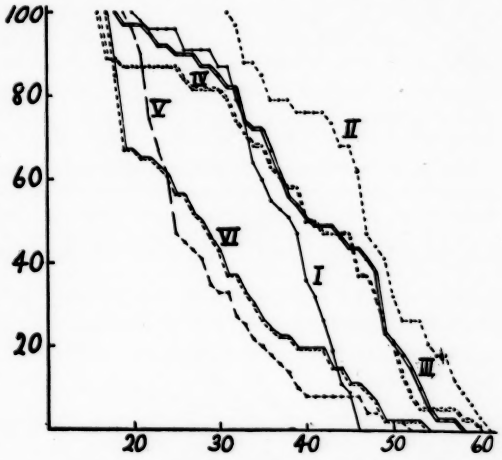

FIG. 2.—Percentage of each group surviving each day, Series II. Ordinates, percentage surviving; abscissas, days of survival. *I* represents well-fed controls; *II*, semistarved controls; *III*, group well fed to third instar; *IV*, well fed to sixth instar; *V*, well fed to twelfth instar; *VI*, well fed to ninth instar.

In comparison with control groups in Series II, there was a high degree of variability with respect to longevity in the groups well fed to the third and to the sixth instars and an intermediate degree of variability in the groups well fed to the ninth and to the twelfth instars. This is shown in Figure 2 where the percentage surviving in each group each day may be read from the survival curves plotted for each group in the series. These curves (for the groups transferred at the sixth, ninth, and twelfth instars—approximately the sixth, twelfth, and eighteenth days, respectively) plainly show that relatively large numbers of animals in the test groups dropped out in a comparatively short time after being changed from normal to dilute medium, but that the animals surviving the change for a few days tended to drop out at a lower rate than was characteristic for controls during the latter part of the experiment.

It will be recalled that a similar result was obtained in Series I with the group well fed to the sixth instar. These considerations led the author to suppose that the very decided decrease in amount of food given these previously well-fed animals in the various test groups at such times as the animals were changed from normal to dilute medium constituted a severe "shock" to the animals which were at such times undoubtedly living at a high metabolic rate, since the normal medium provides approximately optimum conditions. Since considerable numbers in all test groups did survive and since these survivors died off at a lower rate than did normal controls during the latter part of the experiment, it seemed likely that, if normal animals were changed from

normal to dilute medium by stages instead of abruptly, animals thus gradually changed might become "conditioned" against the apparent shock. Such an attempt was made in both Series III and Series IV, in which series were included groups as listed in Table 1. In Series III the "graduated" group was arranged by putting a group of normal animals into one-tenth normal medium at the beginning of the ninth instar; into one-twentieth normal at the tenth; into one-thirtieth normal at the eleventh; and finally into one-fortieth normal at the beginning of the twelfth instar. The dilution used throughout for retarded controls in this series was one-fortieth the normal strength.

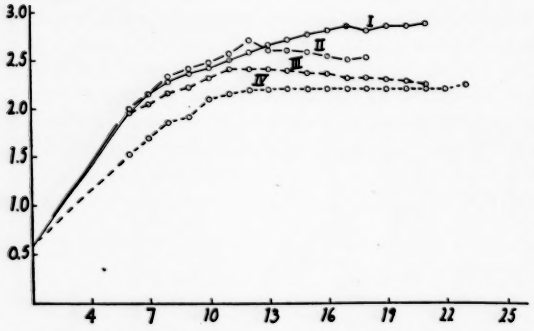

FIG. 3.—Average length of animals in each group at birth and at beginning of each instar after fifth, Series I. Ordinates, body length in millimeters; abscissas, number of instar. *I* represents well-fed controls; *II*, group well fed to twelfth instar; *III*, well fed to sixth instar; *IV*, semistarved control group.

This attempt to condition animals against a sudden change of medium failed, as is evident from an examination of Table 1 (Ser. III). It seemed possible that the earlier steps of the graduating process should be smaller, since one-tenth the normal medium had seemed to have as damaging an effect as did the one-fortieth normal into which the group well fed to the ninth instar was changed abruptly. In Series IV another attempt at conditioning was made, the same as in Series III except that the steps used were one-half normal, one-fourth normal, one-tenth normal, and one-fortieth normal. Table 1 (Ser. IV) shows that this attempt was again futile and that the "conditioned" animals were in most respects about equal to animals well fed to the ninth or twelfth instars and then semistarved.[3]

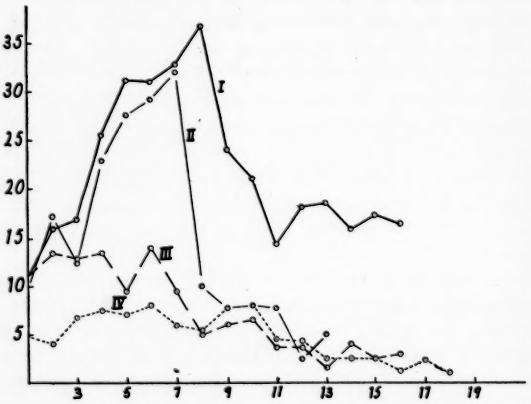

FIG. 4.—Average numbers of young per brood for all groups, Series I. *I* represents well-fed control group; *II*, group well fed to twelfth instar; *III*, well fed to sixth instar; *IV*, semistarved control group. Ordinates, number of young in brood; abscissas, number of brood.

In both Series III and IV large numbers of animals in the groups well fed to the ninth

[3] In the author's opinion neither Series III nor Series IV is very significant, since some difficulty with culture medium was experienced in both series. Even normal controls in these two series were atypical in average longevity, etc., as compared with normal controls in Series I and Series II or as compared with normal animals reared by other workers in this laboratory.

and to the twelfth instars died during the first instar after the change from normal dilute medium. This was also true for the graduated group. For this reason no animals in these groups were considered in the data except those which survived one complete instar after the change. For purposes of comparison the data for control groups was treated similarly in these two series.

BODY GROWTH

The growth of all groups in each series was studied; but, since growth phenomena were much the same in all series, only the data for growth of the four groups studied in Series I will be considered in this paper. The average length of the animals at birth in all four groups was slightly over 0.6 mm. Growth was fairly rapid for all groups until the beginning of the sixth instar, although there was a considerable difference between the growth of animals being maintained in normal medium and the growth of the semistarved control group. As can be seen from Figure 3, the average maximum length attained by the normal animals was slightly over 2.8 mm., a size not attained until the beginning of the sixteenth instar. From the beginning of the twelfth instar the semistarved controls quite regularly measured around 2.2 mm. Neither the group well fed to the sixth instar nor the group well fed to the twelfth instar attained as great an average maximum length (2.4 mm. for the former group, and 2.7 mm. for the latter) as that reached by normal controls. The decrease in average length toward the end of the experiment shown for the test groups in Figure 3 may in part be ascribed to the fact that larger individuals in the two groups had a tendency to die earlier than did smaller ones, but in addition an appreciable number of individuals in these groups showed a positive decrease in length for 1 or 2 instars before death.

SIZE OF BROODS

The wide difference between the average number of young per brood for normal controls and for semistarved controls is apparent in Figure 4. Naturally, the group well fed to the twelfth instar was rather close to the normal controls until the period of starvation began, as was true also of the group well fed until the sixth instar. After the animals in these two groups had been transferred into the dilute medium, they promptly approached a level of reproductive activity about equal to that characteristic of the semistarved control group.

DISCUSSION

Ingle, Wood, and Banta (1937) showed that *Daphnia* which had been semistarved up to the beginning of the twelfth, fifteenth, or even eighteenth instars were physiologically younger animals than normal controls at the same instars, as was apparent from their prompt resumption of rapid growth, increased reproductive activity, and increased longevity. They continued to live for a singificantly long time after both control groups had died. But the results reported here indicate that, when the opposite sort of treatment (well fed earlier and semistarved later) was imposed upon *Daphnia*, only those animals subjected to environments poor in food from the beginning of the third or sixth instars were young enough to be able to adjust themselves to the lowered metabolic rate enforced by such an environment so as to live longer than normal control animals. Animals well fed to the ninth or twelfth instars and then semistarved suffered severely from lack of food, as was evident from the immediate, sharply increased mortality, the marked decline in numbers of young produced, the later negative growth in many individuals, and a somewhat shorter life-span as compared with normal controls.

Well-fed animals undoubtedly age rapidly, for they grow and reproduce at a comparatively high rate. Perhaps the fact that they have aged considerably by the ninth or twelfth instars partly explains the fact that some of them are incapable of becoming adjusted to living at a lower rate of metabolism and thus fail to survive very long when given only a limited amount of food. Such an interpretation leaves unexplained the fact that in some series a number of individuals survived after the change from normal to dilute medium for so long a time as to outlive the longest-lived, normal controls.

Another point to be borne in mind is that animals well fed to the ninth or twelfth instars were considerably larger at these instars than were semistarved controls at these same instars, which, of course, means that these larger animals were composed of a greater mass of protoplasm and required more nourishment. Since they received the same treatment as semistarved controls after the beginning of the ninth or twelfth instars, they must have received less food in proportion to their size than did semistarved controls; hence, they were really more severely starved. A simple calculation based on body-length data taken from Figure 3 will serve to show approximately how great was the size difference at the beginning of the twelfth instar between the animals in the group well fed to the twelfth instar and those in the semistarved control group. The average length of the animals in the former group at this stage was 2.6 mm., while the average length for the latter group was 2.2 mm. In other words, the former figure is to the latter as the ratio 1.18:1.0. When this ratio is cubed, it appears that the average size of the well-fed group at this stage was approximately 1.64 times as great as the average size of the semistarved control animals. It seems reasonable that animals so much larger than semistarved control animals might be rather severely starved while semistarved control animals would not suffer from lack of food; furthermore, the latter animals were already adjusted to the dilute medium.

SUMMARY AND CONCLUSIONS

Longevity of *D. longispina* has been studied for groups of animals well fed to the beginning of the third, sixth, ninth, and twelfth instars and then provided with food in limited amounts (semistarved). Results for these groups have been compared with results for controls well fed throughout life and with results for controls semistarved throughout life. Reproductive activity and growth were also studied for the various groups

The data indicate that, when *Daphnia* are well fed to the beginning of the third instar and then semistarved, the average longevity of the group is about 10 per cent higher than the average for well-fed controls but about 13 per cent lower than for semistarved controls. When *Daphnia* are well fed to the sixth instar and then semistarved, the average longevity of the group is near that for normal controls; and when well fed to the ninth or twelfth instars and then semistarved, the average longevity is lower than for well-fed controls. However, greater variability with respect to longevity is usually observed in these various test groups than is observed in a normal, control group; but maximum length attained and average numbers of young produced per mother are always lower in the test groups than in the well-fed, control group

It seems to make little or no difference whether well-fed animals are changed suddenly from normal to dilute medium or are introduced into the dilute medium by stages.

The suggestion is made that animals well fed to the ninth or twelfth instars and then given a limited supply of food show a decreased average longevity as compared with

normal controls because they have aged considerably by the time the change is made and are therefore incapable of becoming adjusted to a scanty supply of food. It is also pointed out that animals well fed to the ninth and twelfth instars are about 1.6 times as large as semistarved controls at the same stages. Thus, when well-fed animals are given the same food as semistarved controls at these stages, they are in reality starved more severely than are the controls with which they are compared.

The foregoing results, together with those of Ingle *et al.*, indicate that *Daphnia* have relatively short lives if well fed for 9 or more instars and then given only a limited food supply; that *Daphnia* well fed throughout life (normal controls) stand next in order of longevity; that *Daphnia* continuously maintained in limited food live longer than continuously well-fed animals; and that animals maintained in limited food for 9, 12, 15, or even 18 instars and then given abundant food show even greater longevity in direct proportion to the number of instars so maintained.

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STUDIES IN MASS PHYSIOLOGY: EFFECT OF NUMBERS UPON THE OXYGEN CONSUMPTION AND LOCOMOTOR ACTIVITY OF CARASSIUS AURATUS[1]

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RESEARCH on animal aggregations has shown that many physiological processes of animals are controlled or modified by the number of animals mutually participating in these processes. Allee (1931 and 1934) has comprehensively reviewed the work that has been done in this field. "Group effects" have been found for breeding, hibernation, estivation, growth, death-rate, resistance to toxic agents, and other processes. An effect of grouping on oxygen consumption has been demonstrated by Allee (1927), Schuett (1933 and 1934), and Bowen (1932).

Schuett (1933), attempting to bridge the gap between the solitary fishes and those which exhibit highly integrated mechanisms of aggregation, reported that, in a given volume, the oxygen consumption of isolated goldfishes was significantly higher than if in a group of four. Stating that the unmodified Winkler technique he had used for determining oxygen tension was at fault, owing to nitrite contamination, Schuett (1934) repeated his experiments, using the permanganate modification of the Winkler method, and found no significant difference in oxygen consumption between goldfishes in isolation and in groups of four. Schuett (1934) and Escobar, Minahan, and Shaw (1936) pursued the analysis of the problem by determining the effect of grouping on the locomotor activity of goldfishes; they found a significantly higher rate of activity if the animals were isolated than if they were grouped.

It has been the purpose of the present studies to repeat the analysis of this problem and to find the correlation, if any, between oxygen consumption and locomotor activity.

MATERIALS AND METHODS

The goldfish, *Carassius auratus* Linné, was the experimental animal throughout.

The oxygen consumption was determined by means of the modified Winkler technique, as described by Allee and Oesting (1934). The permanganate modification was used to eliminate the error due to nitrite contamination. Samples of water were taken immediately before and after the test period. These were analyzed for oxygen in terms of cubic centimeters of dissolved oxygen per liter of water. The difference, multiplied by the number of liters of water used and divided by the number of fishes in the group, yields the oxygen consumption per fish.

Heavy mineral oil was used as a seal to prevent the leakage of oxygen from the air into the water during the course of the experiments. The oil seal was approximately 3.5 cm. thick. There is the criticism that mineral oil is not a perfect seal and that oxygen will diffuse through into the water and raise the tension. Control experiments show that this is true. The controls were run with oil that had been used from one to seven times

[1] The writer wishes to express his gratitude to Professor W. C. Allee for the suggestion of this problem and for his helpful advice and criticism.

during a period of $2\frac{1}{2}$ weeks. Fishes in isolation, in groups of two, and in groups of four were allowed to bring down the dissolved oxygen content of the water to the average found at the end of the experiments on fishes in these respective groupings. This assures starting with the usual lower tensions used; and the leaks found are nearly or quite the maximum, not minimum, possible under the given conditions, since the oxygen leak is the greater (other things being equal) the lower the amount of dissolved oxygen in the oil-protected water. Results show a leakage range of from 1.15 per cent to 1.55 per cent of the total oxygen available in both 6 and 12 liters in 3 hours. The greatest leakage occurs at tensions found at the end of the experimental period when four fishes are present. This means that the apparent oxygen consumption of the group of four has been decreased by the leak more than that of the group of two or of the isolated fishes. However, the actual difference in oxygen leaks of the various groups is almost negligible and is not sufficient to alter the conclusions reached in these experiments.

The fishes for each day's tests were picked at random from a lot of twenty selected so as to be approximately equal in size. Their length was between 8 and 10 cm. from the tip of the nose to the base of the tail. Animals of this rather large size were used because (1) one such fish consumes a measurable amount of oxygen in 3 hours; (2) a 3- or 4-hour experimental period is less subject to variations—light intensity, for example—than is a longer period; and (3) the shorter the test period the less the oxygen leak through the oil.

Rectangular, assembled aquariums whose maximum capacities were 7.5 and 15 liters of water were used, the smaller when the experimental volume was 6, the larger when the experimental volume was 12 liters of water in all sets of experiments but two, in which the volume was only 6 liters. The dimensions in centimeters of these aquariums were:

Liters	Length	Width	Depth
7.5.....	25	20	13 (to the 6-liter mark)
15.....	30	27	8.5 (to the 6-liter mark) 17 (to the 12-liter mark)

Experiments A, B, C, and D on oxygen consumption were performed at the Hull Zoölogical Laboratory between the hours of 10:00 A.M. and 4:00 P.M. Well water was used at all times. Since the laboratory faces the north, the variations in light intensity were reduced to a minimum during the experimental hours. The temperature range was from 20° to 22° C. Experiments E, F, G, and H on oxygen consumption were performed at the near-by Whitman Laboratory. Since well water was not available, lake water, from which the chlorine and sediment had been removed by filtering through activated charcoal and limestone, was used. Temperature and illumination conditions were approximately the same as at the Hull Laboratory. All experiments performed at Whitman Laboratory were carried out between 12:30 P.M. and 5:00 P.M. The fishes were fed once a week, at the conclusion of a day's experiments, and were not used for at least 20 hours thereafter.

At the end of each day's experiments, the animals were placed into a common aquarium, from which they were picked at random to be placed in isolation, in groups of two, and in groups of four into aquariums containing fresh water through which air was con-

stantly bubbled. These aquariums contained the same volumes of water as were used in the tests the next day. A 20-hour period of acclimatization was allowed in experiments A, B, C, and D and a 21-hour period in Experiments E, F, G, and H. Immediately before each day's tests the air tubes were removed, the water was again changed (except in the "conditioned"-water experiments), and the aquariums were filled 1 liter above the mark. The extra liter of water was added to replace that lost in the process of obtaining three samples of water for analysis at the beginning of the experimental period. These samples were obtained immediately after placing a 3.5-cm. layer of oil on the water. At the close of the test period, samples were again taken and analyzed.

The experiments on locomotor activity were all performed at the Whitman Laboratory under the general conditions mentioned above. The volumes of water, the aquariums, and the feeding and acclimatization techniques were the same as those used in the oxygen-consumption experiments. Also, a 3.5-cm. layer of oil was placed on the water in order to duplicate more completely the general conditions of the experiments on oxygen consumption. In the activity tests the oxygen leak does not enter as a direct factor of error because activity is measured by the observer, not indirectly by chemical analysis. From the twenty fishes used in the oxygen-consumption experiments a group of seven in Experiments A, B, and C and a group of nine in Experiments D, E, F, and G were selected; all the animals selected had distinctive color patterns, so as to be readily identified, and were of approximately the same length.

The technique employed in measuring locomotor activity was that described by Escobar, Minahan, and Shaw (1936) except that all observations were made by one person. Aquariums of the dimensions and capacities listed above were marked off with red India ink into 5-cm. squares on all four sides. Since not all of the dimensions of the aquariums were multiples of five, some of the boxes were rectangles rather than squares. However, movement of a fish into one of these smaller boxes was considered equivalent to movement into the larger squares, since the variable was the same for the various types of grouping. The 5-cm. squares, when viewed in space, constitute a series of imaginary cubes of 125 cc. capacity through which the animals pass when swimming. The eye of the fish was used as the anatomical landmark in judging whether it had passed from one cube into another. The swimming of a fish from one cube into another constitutes a "movement."

There are several sources of error inherent in this method. The greatest lies in the poor estimation of the number of cubes traversed by a rapidly moving animal. When it is moving in several planes at once diagonally across the aquarium, the error is increased. The error can be diminished to some extent by having the guide lines as narrow as possible without obscuring vision.

Another source of error is the fact that fishes may make several movements within a cube; these movements are not recorded in the count. Or they may, by one strong movement, traverse as many as five cubes; and we have no data concerning the oxygen requirements of relatively strong and relatively weak movements which move the animal through the same space. Also, in moving horizontally or vertically from one cube into another, a fish may traverse as many as 9 cm. or as little as 1 cm.

No attempt has been made to differentiate vertical, horizontal, and diagonal movements. The observer is stationed about 2 feet away from the aquarium. It is essential that no sudden noises or movements occur during the observations, as the animals may react violently to them. At no time during the recorded experiments did the fishes

appear to be reacting to such external stimuli. Neither did they give indications of being aware of the presence of the observer. With some practice and the aid of two mirrors placed so as to reflect the narrow sides, the observer facing the long side of the aquarium with his eyes below the level of the water can observe movements in three dimensions, though the accuracy is undoubtedly less than with three observers.

Since the fishes were of distinctive color patterns, individual records could be kept. Each animal was observed for 5 minutes of each experimental hour. Each was placed in a different type of grouping each day so that possible variations might be more evenly distributed. Time was recorded by a stop watch.

OXYGEN CONSUMPTION

Oxygen tension is an important factor in experiments involving oxygen consumption of aquatic animals that utilize dissolved oxygen, and three general categories are recognized (Hyman, 1929): (1) animals completely dependent on the oxygen tension of the water (the lower the tension the less the respiratory rate; the higher the tension the greater the respiratory rate); (2) animals independent of the oxygen tension at the median ranges and dependent at the extremes; and (3) animals independent of the oxygen tension of the medium. Keys (1930) says that no general statements can be made for fishes and that at the asphyxial level their oxygen consumption is very low. Toryu (1927) concludes that *C. auratus* is independent of the oxygen tension of the water between 7.5 and 2 cc. of oxygen per liter. This would indicate that it belongs in class (2).

By correlating the oxygen tension at the end of an experiment with the total oxygen consumption of the fishes, it was possible to determine that the animals in the present set of experiments were independent of the oxygen tension from 7.0 to 1.5 cc. of oxygen per liter. Below this the oxygen-consumption rate was lower and drifted with the tension. The results of the oxygen-consumption experiments listed are those in which the oxygen tension at the conclusion of the experiment was 1.5 cc. per liter or higher.

In Experiments A–D of Table 1, each figure for the oxygen consumption per fish in a group of four is an average of the consumption of the four animals in that experiment. Similarly, the consumption per fish in a group of two is the average of the consumption of the two fishes. Each figure for the oxygen consumption of an isolated animal does not represent an average of four or of two fishes, each in isolation, but is the observed respiration by one. Hence, Experiments A–D in Table 1 do not represent an equal sampling. The fishes in a group of four have had the greatest sampling, and those in isolation the least. Experiments E–H of Table 1 do have equal sampling, since there are four times as many cases listed for isolated fishes as for those in a group of four and twice as many as for those in a group of two.

Tables 1 and 1*a* summarize the effect of grouping on the oxygen-consumption of the goldfish. In general, these tables indicate that isolated fishes consume more oxygen than do those in a group of two or four, and that fishes in a group of two tend to consume the same amount of oxygen as do those in a group of four. In some cases, e.g., B and F in the "I vs. II" column and H in the "I vs. IV" column in Table 1*a*, the differences lack statistical significance. Conditions B and C in the "II vs. IV" column of Table 1*a* indicate that fishes in a group of two have a significantly higher oxygen-consumption rate than do animals in a group of four. The combined experimental means show very good significance for the "I vs. II" and "I vs. IV" relationships, while the "II vs. IV"

relationship shows no statistical significance, though the P value is close to the upper limit of accepted statistical significance.

TABLE 1

EFFECT OF NUMBERS PRESENT UPON THE AMOUNT OF OXYGEN CONSUMED PER FISH BY GROUPED AND ISOLATED GOLDFISHES UNDER EXPERIMENTAL CONDITIONS

Experimental Condition	Test Period (Hours)	I. One Fish		II. Two Fish		IV. Four Fish	
		No. of Cases	Mean Cc. O_2 Consumed per Fish	No. of Cases	Mean Cc. O_2 Consumed per Fish	No. of Cases	Mean Cc. O_2 Consumed per Fish
A[1, 3, 4, 6]	4	12	9.21	15	5.67	10	4.64
B[1, 4, 6]	4	18	8.46	19	8.43	8	5.80
C[1, 5, 6]	4	20	9.45	20	7.28	11	5.69
D[2, 5, 6]	4	34	12.36	34	8.17	24	8.24
E[1, 4, 6]	3	20	7.85	10	5.82	5	5.07
F[2, 5, 6]	3	20	8.76	10	7.02	5	6.18
G[1, 4, 7]	3	12	6.50	6	3.91	3	3.72
H[2, 5, 7]	3	12	7.54	6	3.54	3	4.15
Average of combined means		8	8.77	8	6.23	8	5.44

[1] Six liters of water. [2] Twelve liters of water. [3] Preliminary set. [4] In 7.5-liter aquarium. [5] In 15-liter aquarium. [6] Unconditioned water. [7] "Conditioned" water.

TABLE 1*a*

STATISTICAL ANALYSIS OF TABLE 1 SHOWN IN P VALUES*

Experimental Condition	I vs. II	I vs. IV	II vs. IV
A	.0028	.0020	.0800
B	None†	.0476	.0044
C	.0002	.0000	.0003
D	.0004	.0000	None†
E	.0148	.0160	.1216
F	.0640	.0370	.4000
G	.0040	.0250	.6620
H	.0010	.0950	.4780
A–H combined‡	.0012	.0000	.0566

* Upper limit of significance is set at .05. This is three times the probable error. A P-value of .0010 indicates good significance, while a value of .1000 indicates no significance ("Student," 1925).

† "None" indicates that no significance was calculated, since the means are almost identical.

‡ In the combination of A–H in Table 1*a* and A–G in Table 2*a* it must be noted that in some cases the oxygen consumption and locomotor activity of the fishes are significantly affected by differences in the quantity and quality of the water (see Tables 4–9). However, the combinations are justified since we are dealing with sets of paired data.

LOCOMOTOR ACTIVITY

Tables 2 and 2*a* summarize the effect of grouping on the locomotor activity of the goldfish. The general technique used has been described above. There has been equal

sampling for the fishes in each type of grouping, since individual records have been kept. Each animal has been in each type of grouping, in any given experiment, for the same period of time. The test periods were 3 and 4 hours, with each animal under observation for 5 minutes of each hour of that time.

TABLE 2

EFFECT OF NUMBERS PRESENT UPON THE ACTIVITY OF GROUPED AND ISOLATED GOLDFISHES UNDER EXPERIMENTAL CONDITIONS (RESULTS ARE IN TOTAL AMOUNT OF MOVEMENT* PER FISH PER 5-MINUTE PERIOD OF OBSERVATION)

Experimental Condition	Test Period (Hours)	I. One Fish		II. Two Fish		IV. Four Fish	
		No. of Cases	Mean Activity	No. of Cases	Mean Activity	No. of Cases	Mean Activity
A[1, 3, 5, 7]	4	7	82.9	7	48.0	7	54.8
B[1, 4, 5, 7]	4	7	55.0	7	47.2	7	41.8
C[2, 4, 5, 7]	4	7	76.7	7	48.9	7	45.4
D[1, 3, 5, 7]	3	9	137.8	9	76.1	9	74.9
E[2, 4, 5, 7]	3	9	154.0	9	76.7	9	65.7
F[1, 3, 6, 8]	3	9	136.8	9	81.1	9	61.5
G[2, 4, 6, 8]	3	8	163.5	8	78.3	8	73.9
Average of combined means		7	115.2	7	65.2	7	59.7

* See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."
[1] Six liters of water. [3] In 7.5-liter aquarium. [5] Unconditioned water.
[2] Twelve liters of water. [4] In 15-liter aquarium. [6] "Conditioned" water.
[7] Each case is the average of twelve 5-minute observation periods.
[8] Each case is the average of six 5-minute observation periods.

TABLE 2*a*

STATISTICAL ANALYSIS OF TABLE 2 SHOWN IN *P*-VALUES*

Experimental Condition	I vs. II	I vs. IV	II vs. IV
A	.0170	.0110	.2200
B	.3700	.0094	.3560
C	.0390	.0228	.3700
D	.0204	.0102	.8770
E	.0400	.0220	.1964
F	.0820	.0266	.0758
G	.0678	.0454	.6160
A–G combined†	.0030	.0032	.1700

* See Table 1*a* for discussion of *P*-value.
† See Table 1*a* for discussion of combination of experimental means.

The experiments on locomotor activity were performed to see whether the "group effect," as manifest in the oxygen-consumption experiments, is correlated with the amount of movement performed by each fish. Since, in general, oxygen consumption is directly proportional to activity, the locomotor-activity experiments should give results similar to those on oxygen consumption.

Tables 2 and 2*a* indicate, in general, that isolated fishes have a higher rate of locomotor activity than do those in a group of two or a group of four, and that fishes in a group of two tend to have the same rate of locomotor activity as do those in a group of four. Thus, the results correlate, as they should, with the results on oxygen consumption. In several cases, e.g., B, F, and G in the "I vs. II" column of Table 2*a*, the differences lack statistical significance. The combined experimental means show good significance for the "I vs. II" and the "I vs. IV" relationships, and a *P*-value considerably above the upper limit of accepted statistical significance for the "II vs. IV" relationship.

Since these observations on locomotor activity were made over a period of 3 or 4 hours with an oil seal, and since no Winkler analyses were made for oxygen tension at

TABLE 3

COMPARING THE ACTIVITY OF FISHES IN A GROUP OF FOUR DURING THE FIRST AND LAST HOURS OF THE EXPERIMENTAL PERIOD (RESULTS ARE IN TOTAL AMOUNT OF MOVEMENT* PER FISH PER 5-MINUTE PERIOD OF OBSERVATION)

Experimental Condition †	Test Period (Hours)	First Hour		Last Hour		*P*-Value¶ First vs. Last
		No. of Cases	Mean Activity	No. of Cases	Mean Activity	
A‡	4	7	66.7	7	51.0	.0840
B‡	4	7	44.0	7	37.2	.1800
C‡	4	7	49.7	7	45.0	.4680
D§	3	9	77.1	9	75.2	None**
E§	3	9	64.1	9	63.1	None**
F‖	3	9	65.0	9	55.8	.2480
G‖	3	8	70.9	8	72.2	None**
Average of combined means††		7	62.5	7	57.1	.0348

* See discussions of locomotor activity under "Materials and Methods" for explanation of term "movement."
† Letters correspond to those in Table 2.
‡ Each case is the average of three 5-minute observation periods.
§ Each case is the average of four 5-minute observation periods.
‖ Each case is the average of two 5-minute observation periods.
¶ See Table 1*a* for discussion of *P*-value.
** "None" indicates that no significance was calculated, since the means are almost identical.
†† See Table 1*a* for discussion of combination of experimental means.

the end of the test period, the criticism might be made that the fishes in the group of four might have been reacting to an oxygen tension below 1.5 cc. per liter during the latter part of the test period. If so, the results obtained could be attributed not to a "group effect" but to a low oxygen tension which inhibits respiration and activity.

Accordingly, the movements of fishes in a group of four during the first hour of the test period, when the oxygen tension is the highest, were compared with the movements during the last hour of the test period, when the oxygen tension is the lowest. The results are summarized in Table 3.

In no test was the activity of the fishes significantly higher during the first hour of the test period. However, a consideration of the combined means does show a signifi-

cantly higher rate of activity during the first hour, as compared with that of the last hour. When, however, this is compared with the larger mean differences in Table 2, it can be seen that the factor of inhibiting oxygen tensions is not the principle one at work. In fact, under the conditions of these experiments, it is reasonable to assume that the lower activity during the last hour of the test period indicates a more complete recovery from the effect of handling incident to manipulations near the beginning of the observations, rather than being primarily a result of lessened oxygen supply.

When isolated fishes and those in a group of four are compared, the "group effect" on their oxygen consumption and locomotor activity is apparent. Isolated animals are more active and have a higher rate of oxygen consumption. The only exception to each individual comparison being significant when considered alone is found in H of Table 1*a*, and there, we believe, the lack of significance is due to the small number of tests for the group of four. Supporting this view is the marked significance when A–H are combined.

The group of two seems to occupy the same place as the group of four in the comparison of isolated and grouped fishes. There are more cases where individual experiments fail to show that the higher oxygen consumption and locomotor activity of the isolated animal is significant when compared with a fish in a group of two, but the combined means of all the experiments indicate good statistical significance. In general, the fishes in groups of two tend to have the same rate of oxygen consumption and locomotor activity as those in groups of four. Such results may indicate that the isolated state in the phenomenon is a unique one.

VOLUME RELATIONS

Two important factors that enter into the analysis of the problem are the volume of water available to each animal and the dimensions of the container. Schuett (1933) states that when the volume of water per fish is equal the "group effect" on oxygen consumption disappears. Escobar, Minahan, and Shaw (1936) state that "when a fish is limited to a given volume of water the configuration of that volume or the interrelation between the relative magnitudes of its three dimensions determines to a considerable extent the total amount of movement." They find that when goldfishes in isolation and in groups of four are placed in four different volumes of water—1,625 cc., 3,250 cc., 4,875 cc., and 6,500 cc.—minimal activity in grouped and isolated animals occurs at a volume of 4,875 cc., having a depth of 16.92 cm., a width of 12 cm., and a length of 24 cm. The width and length are constant for all volumes. "The locomotor mechanisms of fishes (with a few exceptions, e.g., seahorse) are adapted to propel the fish along the long axis of its body, the latter being normally oriented in most species of fishes in a horizontal plane," say Escobar, Minahan, and Shaw (1936).

Two questions involved in the volume-relationship analysis are: (1) Does the oxygen consumption and locomotor activity of a goldfish vary with the volume of water available to it? (2) When the volume of water per fish is equal, does the "group effect" on oxygen consumption and locomotor activity disappear? Tables 4–7 attempt to answer these questions, utilizing the data in Tables 1 and 2. Only those data were used with which legitimate paired comparisons could be made.

Table 4 indicates that, in the cases tested, a goldfish has a higher rate of oxygen consumption when in a larger volume of water. Table 6, the corresponding table on locomotor activity, should confirm the results of Table 4 but does not. An increase in volume does not produce an increase in the activity of a goldfish. This contradiction

TABLE 4

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN ISOLATION, GROUPS OF TWO, AND GROUPS OF FOUR IN 6 LITERS WITH THE OXYGEN CONSUMPTION OF SIMILAR COMBINATIONS IN 12 LITERS*

Six Liters		Twelve Liters	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated.....	8.46 7.85 6.50	Isolated.....	12.36 8.76 7.54
Group of two.....	8.43 5.82 3.91	Group of two....	8.17 7.02 3.54
Group of four.....	5.80 5.07 3.72	Group of four....	8.24 6.18 4.15
Mean.....	6.17	Mean.....	7.33

Statistical significance of the difference, .0236

* Figures are based on data in Table 1.

TABLE 5

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN ISOLATION OR GROUPS OF TWO IN 6 LITERS WITH THAT OF FISHES IN GROUPS OF TWO OR FOUR IN 12 LITERS WHEN THE VOLUME OF WATER PER FISH IS EQUAL*

Six Liters		Twelve Liters	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated.....	8.46 7.85 6.50	Group of two....	8.17 7.02 3.54
Group of two.....	8.43 5.82 3.91	Group of four....	8.24 6.18 4.15
Mean.....	6.83	Mean.....	6.22

Statistical significance of the difference, .2838

* Figures are based on data in Table 1.

makes a generalization impossible but may mean that, as used, the oxygen-consumption tests are the more sensitive.

Table 5 shows a disappearance of the "group effect" on oxygen consumption when the volume of water per fish is equal. Table 7, the corresponding table on locomotor activity, again contradicts the results of the table on oxygen consumption. Thus, it indicates that the smaller groups in the smaller volumes are the more active. However, if both Tables 5 and 7 are examined critically, it is found that the isolated fishes have a considerably higher rate of oxygen consumption and locomotor activity than do those

TABLE 6

COMPARING THE ACTIVITY OF FISHES IN ISOLATION, GROUPS OF TWO, AND OF FOUR IN 6 LITERS WITH THE ACTIVITY OF SIMILAR COMBINATIONS IN 12 LITERS*

Six Liters		Twelve Liters	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated	82.9 137.8 136.8	Isolated	76.7 154.0 163.5
Group of two	48.0 76.1 81.1	Group of two	48.9 76.7 78.3
Group of four	54.8 74.9 61.5	Group of four	45.4 65.7 73.9
Mean	83.8	Mean	87.0

Statistical significance of the difference, .4982

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

in the group of two in twice the volume. On the other hand, the fishes in the group of two in both tables have a rate of oxygen consumption and locomotor activity that corresponds closely to that of the animals in the group of four in twice the volume. This again may mean that the isolated state is a unique one and is sufficient in these cases to overcome the handicap of a reduced total volume (the volume per fish being the same) in manifesting its effect on oxygen consumption and locomotor activity. Evidently, the relation of the volume of water per fish and the dimensions of the container to oxygen consumption and locomotor activity is a complex which requires further analysis.

Schuett (1934), using goldfishes 3.5–4.0 cm. in length, found minimal locomotor activity in a 7.5-liter aquarium in a group of four and in a 15-liter aquarium in a group

of eight. Thus, in the smaller aquarium the activity of a group of eight was higher than that of a group of four even though less water was available, per fish, to those in the larger group.

Escobar, Minahan, and Shaw (1936), working with goldfishes in isolation and in groups of four, found not a regular increase of activity with an increase in the volume per fish but a volume at which minimal activity occurred.

No minimal rates of oxygen consumption and locomotor activity were found in the present studies but might be discovered by using larger groups and larger aquaria.

TABLE 7

COMPARING THE ACTIVITY OF FISHES IN ISOLATION OR GROUPS OF TWO IN 6 LITERS WITH THAT OF FISHES IN GROUPS OF TWO OR FOUR IN 12 LITERS WHEN THE VOLUME OF WATER PER FISH IS EQUAL*

Six Liters		Twelve Liters	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated	82.9 137.8 136.8	Group of two	48.9 76.7 78.3
Group of two	48.0 76.1 81.1	Group of four	45.4 65.7 73.9
Mean	93.8	Mean	64.8
Statistical significance of the difference, .0424			

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

"CONDITIONED" WATER

In an attempt to determine whether there was a chemical factor involved in the "group effect," the following procedure was used. The fishes were placed in isolation, groups of two and of four in aquariums in fresh lake water for a 21-hour period of acclimatization prior to the actual test. However, instead of being transferred to fresh lake water again for the tests, as was the procedure in the controls, they were kept in the same water that had been used for acclimatization. This water was called "conditioned" because fishes had lived in it and had added chemical substances to it. The animals used in these "conditioned"-water experiments were fed once a week, but at least 48 hours were allowed to elapse after feeding before any tests were run. According to Allee, Oesting, and Hoskins (1936), "the amount of conditioning is approximately a factor of the number and the size of fishes present, together with the volume of the

water to which they were exposed, and may be indicated by a "conditioning coefficient."[2] In these experiments, on both oxygen consumption and locomotor activity in "conditioned" water, the coefficients were: 15 and 7.5 for an isolated fish in 6 and 12 liters, respectively; 30 and 15 for a group of two in 6 and 12 liters, respectively; and 60 and 30 for a group of four in 6 and 12 liters, respectively.

The controls were run with fishes placed into fresh lake water for the test period. The animals in the "conditioned"-water experiments were transferred to aquariums serving as temporary receptacles, while an oil film was placed on the "conditioned"

TABLE 8

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN "CONDITIONED" WATER WITH THE OXYGEN CONSUMPTION IN UNCONDITIONED WATER*

"Conditioned" Water		Unconditioned Water	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated.....	{6.50† 7.54	Isolated.....	{7.85 8.76
Group of two.....	{3.91 3.54	Group of two....	{5.82 7.02
Group of four.....	{3.72 4.15	Group of four....	{5.07 6.18
Mean.....	4.89	Mean.....	6.78

Statistical significance of the difference, .0030

* Figures are based on data in Table 1.

† Each figure in the "conditioned" column represents the same volume of water as does each corresponding figure in the unconditioned column.

water. They were then replaced into the test aquarium. This procedure assured an equal effect of handling in both controls and experimentals.

The "group effect" in "conditioned" water is listed under G and H in Tables 1 and 1*a* and under F and G in Tables 2 and 2*a*. The controls are E and F in Tables 1 and 1*a* and D and E in Tables 2 and 2*a*. In general, the results for "conditioned" water coincide with those for unconditioned water as far as the "group effect" is concerned. The isolated animal is found to be the most active and to have the highest rate of oxygen consumption. Tables 8 and 9 contain a direct comparison of oxygen consumption and locomotor activity of fishes in the same types of grouping in "conditioned" and unconditioned waters.

Table 8 indicates that fishes in "conditioned" water have a significantly lower rate

[2] The conditioning coefficient is obtained by dividing the product of the number of conditioning fishes times their length in millimeters by the number of liters of water conditioned (Allee, Oesting, and Hoskins, 1936).

of oxygen consumption than do those in unconditioned water. The explanation of this result is not clear at this time. Table 9 on locomotor activity shows no difference in

TABLE 9

COMPARING THE ACTIVITY OF FISHES IN "CONDITIONED" WATER WITH THE ACTIVITY IN UNCONDITIONED WATER*

"Conditioned" Water		Unconditioned Water	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated.....	136.8‡ 163.5	Isolated.....	137.8 154.0
Group of two.....	81.1 78.3	Group of two....	76.1 76.7
Group of four.....	61.5 73.9	Group of four....	74.9 65.7
Mean.....	99.2	Mean.....	97.5

Statistical significance of the difference, .6718

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

‡ Each figure in the "conditioned" column represents the same volume of water as does each corresponding figure in the unconditioned column.

TABLE 10

EFFECT OF NUMBERS PRESENT UPON THE AMOUNT OF OXYGEN CONSUMED BY GROUPED AND ISOLATED GOLDFISHES*

Isolated		Group of Four		Mean Difference	*P*-Value†
No. of Cases	Mean Cc. of O_2 Consumed per Fish	No. of Cases	Mean Cc. of O_2 Consumed per Fish		
40×1	4.60	10×4	3.22	1.38	.0009

* Test period is 3 hours. Volume is 6 liters of unconditioned water in a 7.5-liter aquarium.

† See Table 1a for discussion of *P*-value.

activity between fishes in "conditioned" and unconditioned water. Again this may mean that, as used, the oxygen-consumption tests are the more sensitive.

PARTIAL RECHECK OF RESULTS, USING MANOMETRIC METHOD

Since it was thought that even the modified method of Winkler, used in determining dissolved oxygen in the oxygen-consumption tests, might possibly contain some hidden error, it was decided to repeat some of the tests, using the Van Slyke manometric method (Van Slyke and Neill, 1924; Oesting, 1934). Allee and Oesting (1934) and Wilder (1937) tested the relative accuracy of the modified Winkler and Van Slyke methods and found agreement.

The materials and methods were the same as those described for the oxygen-consumption experiments using the modified Winkler method. Experimental conditions were identical with those of the earlier series. The oxygen consumption of an isolated fish was compared with that of one in a group of four. The results are summarized in Table 10. The statistical significance of the results is high and shows again that, other things being equal, the oxygen consumption of an isolated fish is higher than that of one in a group of four; this confirms results listed in Table 1. Thus, in so far as tested, findings obtained with the modified Winkler method are confirmed by the Van Slyke manometric method. This agrees with the experience of Wilder (1937).

DISCUSSION

The explanation of the effect of grouping on the respiration and locomotor activity of goldfishes is still hypothetical. Schuett (1933) dismissed the possibility of oxygen tension exerting any effect, at least until the asphyxia level is reached. Toryu (1927) reached the same conclusion, and our own experience is similar. Schuett (1933) also dismissed the possibility that the increased production of carbon dioxide by grouped fishes was the responsible factor.

Escobar, Minahan, and Shaw (1936) offer the suggestion that an isolated fish has no obstruction in its path of motion and can continue along this path undisturbed. On the other hand, a fish in a group must constantly make adjustments to the presence of its neighbors. Though isolated fishes, when active, usually swim in one unobstructed plane, their activity is less constant, and much of it is composed of a series of sudden spurts. Grouped fishes usually swim in several planes; but their activity is much more constant, since they are constantly subjected to stimuli from others in the group.

Vision as an important integrating factor for aggregations of fishes has been demonstrated by Bowen (1931). She reported that blinded catfishes of the species *Ameiurus melas*, which in contrast to *C. auratus* is a more definitely schooling animal, did not aggregate and that normal fishes did not respond in the dark. Bowen (1931) also found that responses to touch are of great importance and that the resulting stimulus may perhaps be the fundamental cause of close aggregations, with vision serving only as a means by which the fishes find one another. Bowen (1932) showed that catfishes in groups of four have a greater rate of oxygen consumption per fish than do those in isolation. This is what would be expected from a closely schooling fish when the individuals push actively toward the center of the group.

Vision may be acting as a factor inhibiting the activity of grouped goldfishes. If so, provided other factors are not exerting an important effect at the same time, blinded goldfishes and goldfishes kept in the dark should not manifest a "group effect." Experiments are now in progress in our laboratory to explore this possibility. It may be that sight reactions will account for all of the observed phenomena of "group effect" in the

goldfish. However, it is possible that vibrations of tails of fishes set up pressure disturbances detected perhaps by lateral line organs, or that other sense organs are involved, either directly or indirectly. Planned experiments include an analysis of these factors.

Our results on oxygen consumption confirm those obtained by Schuett (1933) when there was an error in his technique, and contradict his later results (1934) when the error had been corrected. Schuett (1934) did find a higher rate of locomotor activity for goldfishes in isolation than for those in groups of four. This result does not correlate with his failure to find a higher rate of oxygen consumption in the isolated fish, using the modified Winkler technique. Schuett's experiments on locomotor activity were performed in 7.5- and 15-liter aquariums as were our experiments. His experiments on oxygen consumption were performed in Erlenmeyer flasks; and Schuett offers a possible explanation of contradictory results, stating:

> This may be because of the fact that the respiratory chambers used in oxygen consumption work are of such a size as to produce a crowding effect upon the contained fishes, causing movements that would not otherwise occur. In such experiments the fishes were seldom quiet in the respiratory chambers, usually moving in vertical paths from bottom to top in the chambers. This was true both with isolated fishes and with groups of four. Under these circumstances the effect of quieting, as usually noticed in the groups, was lost as the results here indicate.

Our results confirm those obtained by Schuett (1934) and by Escobar, Minahan, and Shaw (1936) on the locomotor activity of isolated goldfishes and goldfishes in groups of four; and, volume comparisons excepted, a correlation has been established between oxygen consumption and locomotor activity.

It is appropriate here to compare the relative sensitivities of the Winkler chemical analysis used in determining the oxygen consumption and the observational technique used in determining the locomotor activity. In favor of the chemical technique it may be said that it detects two cases, B and C in Table 1, in which the oxygen consumption of the fishes in the group of two is significantly higher than that of the fishes in the group of four though, in general, the oxygen consumption of the two groups tends to be the same. The locomotor activity technique detects no difference in a similar comparison. Table 4 shows a significant increase in oxygen consumption of goldfishes placed in larger volumes, while Table 6 shows no corresponding effect on locomotor activity. Table 8 indicates a decreased oxygen consumption of fishes in "conditioned" water, while Table 9 shows no corresponding effect on the locomotor activity. In favor of the observational technique it may be said that it indicates more effectively in Table 7, comparing the isolated fish in 6 liters with the fishes in the group of two in 12 liters, that the isolated state may be sufficient to overcome the handicap of reduced total volume and to cause greater activity in the same volume of water per fish than does a similar comparison in Table 5 on oxygen consumption. Also, after Schuett (1934) had failed to obtain a "group effect" on oxygen consumption, using the modified chemical technique, he did get a "group effect" on locomotor activity, as did Escobar, Minahan, and Shaw (1936). However, the use by Schuett (1934) of Erlenmeyer flasks instead of aquariums for the tests on oxygen consumption must be borne in mind as a possible explanation. All things considered, it is the opinion of the writer that the chemical technique is the more sensitive one.

It is too early to attempt to discuss the broader implications of these and other studies on the mass physiology of the goldfish. In addition to the problems here at-

tacked, other workers in this laboratory have found (*a*) that when exposed to toxic amounts of colloidal silver, grouped goldfishes survive longer than those in isolation (Allee and Bowen, 1932); (*b*) that in an appropriate, simple setup, grouped goldfishes learn more rapidly than isolated animals (Welty, 1934); (*c*) that goldfishes consume more *Daphnia* per unit of time if grouped than if isolated (Welty, 1934); and (*d*) that under many experimental conditions small, isolated goldfishes grow more rapidly in water in which other goldfishes have lived than in uncontaminated water (Allee, Bowen, Welty, and Oesting, 1934; Allee, Oesting, and Hoskins, 1936; Livengood, 1937). In time, perhaps these and other studies may permit a critical examination of the general survival-value of numbers of goldfishes. For the present it is useless to do more than to recognize that such general problems exist and that they are not dissociated from the present studies.

SUMMARY

1. In a given volume of water an isolated goldfish consumes more oxygen and has a higher rate of locomotor activity than does each fish in a group of two.

2. In a given volume of water an isolated goldfish consumes more oxygen and has a higher rate of locomotor activity than does each fish in a group of four.

3. In a given volume of water each goldfish in a group of two tends to consume the same amount of oxygen and to have the same rate of locomotor activity as does each fish in a group of four.

4. Fishes in "conditioned" water exhibit the same type of "group effect" as do those in unconditioned water.

5. Fishes in "conditioned" water consume less oxygen than do those in unconditioned water.

6. Volume relationships are complex and require further analysis.

7. The modified Winkler technique appears to be more sensitive in determining oxygen consumption than the observational technique in determining locomotor activity.

8. A partial recheck of oxygen-consumption experiments, using manometric methods, confirms the relationships summarized in conclusion No. 2.

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FETAL RESPIRATORY MOVEMENTS IN THE CAT

(Two figures)

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FETAL respiratory movements have been described in several mammals, including the rat (Corey, 1932), rabbit (Snyder and Rosenfeld, 1937), sheep (Barcroft and Barron, 1936 and 1937), and man (Ahlfeld, 1905; Windle, Dragstedt, Murray, and Green, 1938). The present study in the cat was undertaken to compare the movements of respiration at various stages in fetal life and under conditions as nearly normal as possible, as well as in states of experimental fetal anoxemia and carbon dioxide excess. We wished to learn whether or not the fetus responds to conditions which serve as respiratory stimuli in the adult.

Cats in various stages of pregnancy were anesthetized with ether before ligating the carotid arteries high in the neck and the basilar artery at the rostral one-third of the pons (Pollock and Davis, 1924) an hour or more (usually 2 or 3 hours) before experiments were begun. With this procedure the animals entered a state of partial decerebration. Conscious voluntary activity was lost; but respiration, although temporarily impaired by the procedure, was usually restored within an hour or two to about normal and, in several instances, was considerably increased in rate and depth; body temperature and blood pressure were usually not affected significantly. The explanation for the maintenance of rapid breathing and normal temperature seems to be that enough blood reached the midbrain and diencephalon by collaterial circulation around the ligature on the basilar artery to support the integrity of centers there even though the cerebral hemispheres were quite lacking in blood flow. After the vessels had been ligated, ether was discontinued; later on, the cat's abdomen could be opened under physiological saline or Locke's solution in a constant-temperature bath with no further anesthesia. All the fetuses exposed were delivered through small openings in the uterus at the ends of the zonary placentas and were examined with placental circulation intact. Various experimental procedures were used during the course of the study of fetal reactions.

RESULTS IN SPECIMENS OF LESS THAN 40 DAYS' GESTATION

In a series of fifty embryos and small fetuses 13.5–30 mm. length (not listed in Table 1), we observed no rhythmical movements of respiration. However, in the largest (those of 25–30 mm.), occasional and nonrhythmical contractions of diaphragm, abdominal, and intercostal muscles occurred. Some time after exposing the specimens, these movements began to take place with simultaneous extension of head, trunk, and extremities. These integrated activities seemed to be the precursors of truly rhythmical respiration but were brought on by impairing the fetal oxygen-exchange mechanism in delivering the specimens.

[1] Aided by a grant from the American Academy of Arts and Sciences.

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Rhythmical respiratory movements were encountered in all the 122 cat fetuses listed in Table 1, but the nature of the movements varied with the age and size of the specimens and with the physiological conditions obtaining at the time of the experiments. Fetuses younger than about 40 days of gestation (32–58 mm. C. R. length) could usually be exposed in the constant-temperature bath and observed for a few minutes before we noticed the marked changes in the blood color which would signify anoxemia. In none of these specimens viewed under the best of conditions did we observe rhythmical respiratory movements. The specimens often showed movements of the head, limbs, trunk, or tail; but these activities appeared to be random and nonintegrated; the fetuses

TABLE 1

EXPERIMENTS IN WHICH RHYTHMICAL RESPIRATORY MOVEMENTS OF CAT FETUSES WERE STUDIED

Measurements are crown-rump length of fresh specimens. Age is in time after observed matings, except where marked with an asterisk (*), which indicates computation from an age-length curve.

Cat No.	Living Fetuses	Fetal Size		Fetal Age (Days)	Cat No.	Living Fetuses	Fetal Size		Fetal Age (Days)
		Length (Mm.)	Weight (Gm.)				Length (Mm.)	Weight (Gm.)	
1	3	32	.....	33	17	3	80	.....	44
2	6	32–35	.....	32	18	6	80	.....	?
3	4	35	2.6–3.0	32–33*	19	5	85–90	28–33	?
4	4	35	.....	32–33*	20	4	85–90	.....	?
5	4	38–40	.....	33–34*	21	4	95	36–42	?
6	3	38–40	.....	33–34*	22	3	91–101	14–17	?
7	4	40	.....	34*	23	1	100	.....	51
8	3	48–50	.....	?	24	2	100	.....	?
9	5	45–52	.....	?	25	5	100–104	38–46	?
10	5	54–56	.....	39	26	5	110–15	.....	53
11	4	55–58	.....	?	27	4	115	70–76	?
12	4	65–70	.....	44	28	3	120	.....	?
13	4	70–72	.....	?	29	3	135	.....	62
14	4	60–75	.....	?	30	3	140	.....	63
15	4	75	18–19	?	31	4	140	.....	65
16	2	75–80	15–20	?	32	4	140–46	107–27	?

were definitely apneic in respect to respiratory movements. Invariably they began to show respiratory movements a few minutes after delivery, and these soon became rhythmical. This change in behavior appeared to be correlated with constriction of the uterine vessels as the uterine muscle tightened upon the placenta. When left undisturbed in the bath, fetal respiration increased in rate and depth but seldom became constant. Instead, it was intermittent, and the periods of breathing movements seemed to correspond to times when the fetal blood color was somewhat darkened.

The first rhythmical respiratory movements to manifest themselves upon exposure of the fetuses were usually quite delicate, quick, unsustained contractions of the diaphragm, intercostal, and abdominal muscles, and, in a few instances apparently, of the diaphragm alone. With prolonged exposure, more and more muscles shared in this reaction until each "breath" appeared to be a gasp involving extension of the head and

limbs. These violent gasplike respirations never occurred in fetuses whose umbilical veins were of good color. Even when the exposed fetuses were executing marked rhythmical respiratory movements, those *in utero* showed no such activity but could often be seen to move the feet, tail, or head arhythmically.

It was impossible to avoid the implication that fetuses less than 40 days old (58 mm.) were apneic in decerebrated cats and that respiration came about with a state of partial anoxemia which could not be avoided in exposing the fetuses experimentally. To test this further, we clamped the umbilical cords of fetuses immediately after exposing them. Half a minute or more usually elapsed before results appeared. Some of the fetuses executed a few rapid and rhythmical contractions of the diaphragm, usually with concomitant action of intercostal and abdominal muscles; other quick, jerklike movements were often seen. Within a few seconds to half a minute lively squirming (tonic mass convulsive movements) and running movements were observed. Irritability to mechanical stimulation then began to decrease, and muscle tonus increased; the fetus slowly assumed a posture resembling decerebrate rigidity, owing to sustained contraction of trunk and limb muscles. This sustained contraction was interrupted by rhythmical gasps which had begun during the phase of active squirming. Gasping was the last motor phenomenon to stop as death approached. At first the head extended with each gasp; but as depression came on, this changed to flexion. Even after all irritability was lost and when the last feeble gasp had occurred, re-establishment of the placental circulation sometimes led to partial recovery. Occasionally the motor responses were imperfectly re-enacted during recovery, but seemingly in the reverse order of their manifestation during the asphyxia. The phenomena of asphyxia could usually be repeated by clamping the umbilical cord again. Fetal apnea was not permanently resumed after asphyxia; the fetuses usually showed interrupted respiratory rhythms, which seldom involved the delicate movements of the primary breathing muscles alone, until the end of the experiment.

Administration to the mother cats of mixtures of 5–8 per cent oxygen in nitrogen by gas machine and tracheal canula brought about cyanosis, indicated by darkening of the uterine vessels, and respirations of previously apneic fetuses started promptly. It was evident that the changes in behavior observed under asphyxia induced by oxygen lack resembled, but in exaggerated form, those seen when the fetuses were exposed in the bath for prolonged periods; and we concluded that the delivery of the fetus had, indeed, set up a state of partial asphyxia which brought about fetal respirations.

We tested the effects upon fetuses of carbon dioxide given to the mother cats in mixtures of 5–20 per cent in oxygen. With the animals under the influence of 8–10 per cent carbon dioxide the fetuses which had been apneic or showed only an occasional arhythmical respiratory movement when handled at delivery began to execute lively rhythmical respiratory movements, as is illustrated in Figure 1. In other fetuses of the same litters, but which had been exposed for some time, the existing respiratory movements became more rapid and the depths of each increased. Furthermore, even in the smallest so tested (35 mm.), the unexposed fetuses *in utero*, in which we could neither palpate nor see respiratory movements before giving the gas, began to execute rhythmical respirations when the cat breathed the oxygen–carbon dioxide mixture. The color of the umbilical veins and of the fetuses themselves was very good before and during the time the mixtures were given, and there was no visible indication of asphyxia. When the mother cats whose fetuses were apneic were overventilated with pure oxygen

there was no observable stimulation of respiratory activities in the fetuses. We concluded that the carbon dioxide had acted in its usual capacity to stimulate respiration.

Prolonged breathing of high concentrations of carbon dioxide (15–20 per cent) in oxygen led to fetal gasping and depression of the delicate rapid respiratory rhythm; but when air or oxygen was substituted, a rebound stimulation of the respiratory rate like that seen in bird fetuses (Windle and Nelson, 1938) sometimes ensued. In a few experiments the cat fetuses resumed their apneic state for brief intervals after the effects of carbon dioxide had subsided.

RESULTS IN SPECIMENS OF 62–65 DAYS' GESTATION

Gestation in several cats which were mated in our laboratory lasted 64 days. In the hope that new corpora lutea would inhibit uterine contractions (Snyder and Rosenfeld, 1937), attempts were made to bring about ovulation in two cats by administration of pregnancy urine concentrate (Parke Davis' Antuitrin S.) 6 days before the end of gestation. One cat (No. 31) ovulated, apparently on the sixty-fourth day; and the fetuses were studied the following day. Results in this animal were similar to those in the other three cats and will be described with them. The uterus was quite flaccid for the first half-hour.

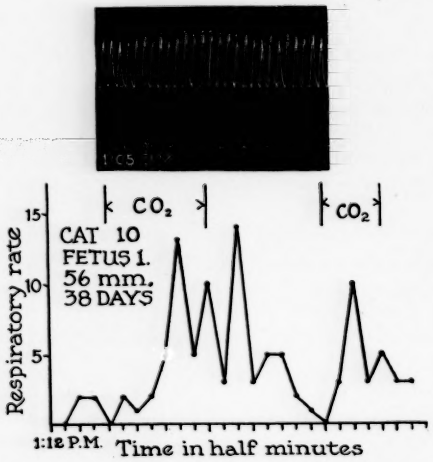

FIG. 1.—The kymograph record illustrates maternal respiration of the cat whose carotid and basilar arteries were ligated at 10:30 A.M., taken just before the animal was placed in the bath and abdomen opened; time in 5-second intervals. The graph illustrates production of fetal respiration at 38 days' gestation by administering 8–10 per cent carbon dioxide in oxygen to the mother cat through tracheal canula. The lag is caused in part by the time it took to get the gas through the delivery tube of the machine; this factor was later controlled by compressing the bags just before administering the gas (see Fig. 2).

The observations of fetal respiratory movements in the fourteen fetuses of the cats of 62–65 days' gestation (fetuses 135–146 mm. long) differed significantly from those in the fetuses less than 40 days old. In the first place, after the effects of ether used during the tying of carotid and basilar arteries had subsided, intrauterine activities of a rhythmical nature could be palpated through the intact maternal abdomen in two of the animals. These were very inconstant, occurring at long intervals as short series of rapid beats. They suggested to the observers similar phenomena encountered after opening the abdomen and which were unquestionably respiratory movements.

Intrauterine respiratory movements were seen in all four animals after opening the abdomen, and were likewise of short duration and occurred at intervals. Upon exposing the fetuses through openings in the uterus made at ends of the zonary placentas, the umbilical-vein blood was found to be very dark and the fetuses seemed to be quite depressed. After the specimens had been exposed with placental circulation intact for

several minutes, they usually began to execute rhythmical respiratory movements, which varied in rate from about 10 to 30 or more per minute and occurred intermittently. The intermittent occurrence of the fetal respiratory rhythm appeared to be associated with contraction of uterine musculature and accompanying improvement in fetal blood color. When a fetus was delivered in the relaxed phase of the uterus, its umbilical vessels were dark and it was apneic. Contraction of the uterus brought about a reddening of the blood in the umbilical veins, and the fetal color improved. As the contraction was subsiding, fetal respiratory activity began and persisted until the umbilical veins darkened again. This series of events occurred in slow rhythm of less than once to as many as four times a minute. When a fetus was delivered from the uterus during a strong uterine contraction, its umbilical veins were somewhat redder and it was executing rhythmical respiratory movements by the time we could get it out. The better the color of the umbilical veins, the more rapid was the fetal respiratory rate and the fewer the muscles involved in each "inspiration." However, delicate respiratory movements, such as were seen in young fetuses, were never encountered in those near term; all involved more muscles than the diaphragm alone.

In none of the cats of 62–65 days' gestation were we able to increase the rate of intrauterine fetal respirations by administering oxygen–carbon dioxide mixtures to the mother cat, nor were we able to start fetal respirations in the apneic exposed fetuses except by bringing about uterine contractions and reddening of fetal blood. On the contrary, mixtures of 5–15 per cent carbon dioxide and oxygen served only to deepen the inspirations, after which they stopped entirely. When oxygen alone was forced upon the cat, respiratory movements of the fetus sometimes began or the rate increased.

These experiments seem to show that fetuses near term, quite unlike those earlier described, were actually somewhat depressed and in a state of partial anoxemia. Conditions which prejudiced their placental exchange mechanism still more did not bring about rapid rhythms of respiratory movements. Conditions which tended to relieve the anoxemia did bring about breathing rhythms. The rhythmical contraction of the uterus itself was a factor acting to relieve the anoxemia intermittently, at least in those specimens which had been delivered with placentas still intact.

RESULTS IN SPECIMENS OF 40–60 DAYS' GESTATION

As might be expected from a consideration of the remarkable differences in results obtained in young and old fetuses of the cat, the observations in specimens of intermediate age were subject to considerable variation. Taken alone, these would prove to be most confusing. With physiological conditions better in some animals than in others, it was to be expected that we would find differences in the degree of oxygenation of the various litters of fetuses. Judging from fetal blood color, this is essentially what we observed. Furthermore, removal of a large fetus from a greatly distended uterus is followed by alterations in the placental exchange mechanism which are much more pronounced than after removal of a smaller fetus, the placentas in the two instances being much the same size. Since we cannot summarize all the experiments of this group in a single description, we propose to illustrate the principal variations.

In cat No. 17, whose fetuses measured 80 mm. in length, conditions appeared to be as good as could be expected. The basilar and carotid arteries had been tied under ether between 9:45 and 10:15 A.M., after which the anesthetic was discontinued; the animal was kept at rest until 1:15 P.M., when the abdomen was opened in the constant-

temperature bath. The cat developed some decerebrate rigidity, but there was no depression of its respiration, which, on the contrary, was rapid and deep. Its temperature was unaffected. During the first 8 minutes after delivering the first fetus there were no rhythmical fetal respiratory movements (Fig. 2); the cat was breathing pure oxygen much of the time. Four respirations occurred without distinct rhythm in the first half-minute after exposing the fetus, and were probably due to handling the fetus; otherwise, only an occasional slight, local movement of the diaphragm with elevation of the costal margin was seen. The undelivered fetuses *in utero* were quite apneic. Ad-

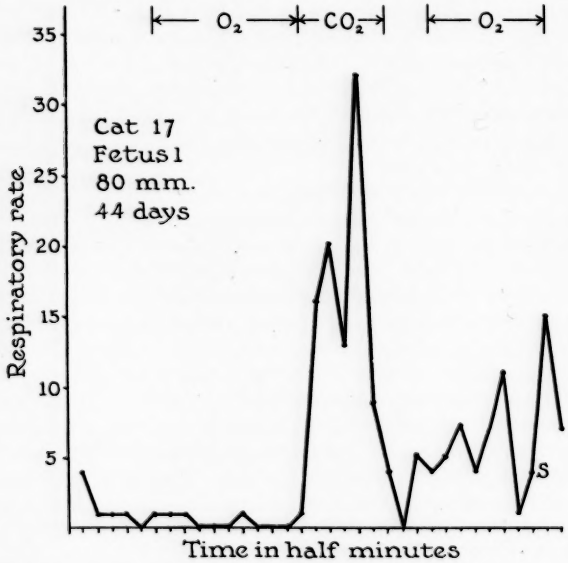

FIG. 2.—Illustrates production of fetal respiration at 44 days by 8–10 per cent carbon dioxide in oxygen. The percentage of carbon dioxide delivered by the machine was controlled for all experiments by sampling and analyzing by the Van Slyke method.

ministration of 8–10 per cent carbon dioxide in oxygen to the mother cat brought about marked respiratory acceleration in the mother cat and simultaneously respiratory movements began in all the fetuses. The respirations of the delivered fetus quickly reached a maximum of 32 in half a minute, then became very deep, involving all respiratory muscles, and began to subside as though the gas were exercising a secondary depressing effect. With oxygen alone following this, fetal respirations again improved suggesting that a certain amount of rebound stimulation occurred as the carbon dioxide excess was being removed. In this experiment the results obtained were like those described in the small fetuses and differed from those observed in most of those 80–120 mm. in length, whose respiratory behavior was much more like that of the larger specimens near term.

It was usually found that 80–120 mm. fetuses executed rhythmical respiratory move-

ments after delivery with placentas intact. These occurred at the conclusion of each rhythmical uterine contraction and showed improvement when the cats were overventilated with oxygen. In some experiments carbon dioxide–oxygen mixtures served to increase rate, as well as depth, of fetal respirations; but it was seldom entirely clear that the increase in rate was not due to an increased oxygenation of the fetuses accompanying augmentation of uterine activity. The first delivered 100-mm. fetus of cat No. 23 illustrates the point in question. The fetus was quite anoxemic when first exposed, and rhythmical respiratory movements were not stimulated when the mother cat was allowed to rebreathe the air in the tube to the gas machine for 4 minutes. But administration of a mixture of 5–8 per cent carbon dioxide in oxygen 6½ minutes after exposing the fetus led to rhythms of fetal respiratory movements reaching 52 per minute. The condition of the mother cat of this experiment seemed to be satisfactory. Perhaps the removal of so large a fetus from a distended uterus had led very suddenly to a condition of anoxemia in the fetus. The oxygen–carbon dioxide mixture apparently stimulated uterine activity, which helped overcome the anoxemia by delivering oxygenated blood to the fetus intermittently.

When experiments were impaired by poor maternal respirations, by increased body temperature, or by decreased maternal blood pressure after ligating the carotid and basilar arteries, the results obtained in the fetuses, even in earlier stages of pregnancy, resembled those recorded under good conditions in fetuses near term. In cat No. 8 (fetuses 48–50 mm. long) experimental conditions after tying carotid and basilar arteries were not as good as usual, and the maternal respiration had to be strengthened by allowing the cat to breathe the oxygen–carbon dioxide mixture for about 5 minutes before we opened the abdomen. The fetuses showed no respiratory movements of the rhythmical type, but they did gasp occasionally. The specimens seemed to be depressed to the point that they could not be stimulated by the carbon dioxide breathed by the mother. When the cat was forced to breathe oxygen alone, rhythmical respiratory movements of the fetus began, reaching a maximum of 34 per minute. It would be hazardous to conclude that results of this nature were representative of normal activities *in utero*. All the experimental conditions must be carefully evaluated.

DISCUSSION

The experiments in cat fetuses of various ages throw some light upon the subject of prenatal respiratory activity and also bring up several questions. We should like to know something more definite about the relationship between fetal and placental blood oxygen and carbon dioxide and fetal respiratory movements. What concentrations of these gases are compatible with the phenomena in question? Such studies have been started. The relationship between uterine contraction and the intermittent occurrence of prenatal respiratory rhythms must be investigated further before we can complete the picture of fetal activities *in utero*. Furthermore, the present studies have demonstrated the importance of correlating observations on the physiological conditions of the maternal animals with the responses obtained in the fetuses. This must receive even greater attention in the future.

There can be little doubt that cat fetuses under normal physiologic conditions execute rhythmical respiratory movements *in utero* in the later days of gestation. Our own observations add evidence to that presented by Ahlfeld (1905) and by Snyder and Rosen-

feld (1937). We believe that this activity is manifested in the cat because the fetuses are experiencing a physiologic anoxemia *in utero*. How extensive such an anoxemia is cannot be told until analyses of the fetal blood have been made under satisfactory experimental conditions. If we may judge at all by color of the umbilical veins and the fetuses themselves, it would appear to be rather marked.

If the cat fetuses near term were already profoundly anoxemic *in utero*, it could not be expected that they would react to conditions of asphyxia or carbon dioxide excess with an increase in respiratory rate. On the contrary, such conditions should lead to further depression. This is what our experiments demonstrated. It is what Snyder and Rosenfeld (1937) found in rabbits at term and in those whose gestation had been prolonged up to 35 days by causing new corpora lutea to be developed before the end of the period and thus inhibiting uterine contractions. It may be significant that Koff and Davis (1937) have demonstrated in rabbits whose labor was inhibited by such methods that fetuses did not live beyond the thirty-sixth day. Perhaps they died of asphyxia. We observed that, during complete flaccidity of the cat's uterus, the fetuses were quite depressed and their blood color was dark. But when contractions of the uterus took place, color improved and the specimens were aroused to the point of executing rhythmical respiratory movements. Are the prelabor contractions of the uterus physiological, and do they serve the very useful purpose of preventing asphyxia in the fetus?

Our experiments in the younger cat fetuses (40 days and less) demonstrated a condition quite different from that obtaining near term. These small fetuses were apneic under normal physiological conditions, but rapid and delicate fetal respiratory movements of a rhythmical nature could be initiated by administering carbon dioxide to the mother or by causing asphyxia of the fetuses. It is reasonable to suggest that observers who have been unable to demonstrate such a stimulating or augmenting action of carbon dioxide have been dealing with specimens already stimulated in a state of anoxemia.

It is impossible for us to say how late in the gestation period fetuses normally are apneic *in utero*. Perhaps as late as the forty-fourth day (80 mm.) or longer. It must depend upon the time in the prenatal life when a physiologic state of anoxemia begins to manifest itself, and this may be an intermittent phenomenon subject to variation.

Observations were obtained in the chick (Windle and Barcroft, 1938; Windle, Scharpenberg, and Steele, 1938) and duck (Windle and Nelson, 1938) which were similar to those in the cat. It was found that carbon dioxide stimulated fetal respiration during the greater part of the life in the egg and that only when normally occurring respirations had attained a rapid rate at the time the respiratory allantois ceased to function efficiently did it begin to act as a depressing factor.

Although we have attempted to gain an understanding of the intrauterine respiratory movements obtaining normally, we have not considered the relationship of the nervous system to these phenomena in the cat fetuses in the present investigation. As Barcroft and Barron (1937) have demonstrated for the sheep, the fetuses' own higher nervous centers seem to play important parts in inhibiting fetal activity including respiration. It is quite possible that such factors come into play at relatively different times in the cat and sheep. In the cat fetuses of 32 days' or more gestation which appeared to be apneic *in utero* and when first delivered, inhibitory influences of the brain may already be at work. We know that descending tracts from the tegmentum and mesencephalic tectum are well formed much before the middle of fetal life (Windle, 1935). This sub-

ject will receive more consideration at a later time. In respect to humoral factors influencing respiratory activities, it is evident, from the present studies, that cat fetuses react very much as do adult animals to the usual respiratory stimuli.

CONCLUSIONS

Cat fetuses tended to be apneic *in utero* during the middle third or more of the gestation period, but factors leading to asphyxia served to inaugurate delicate rapid rhythms of respiration movements.

Severe and prolonged asphyxia depressed the delicate movements, but gasping occurred and persisted after finer movements and irritability to mechanical stimulation were lost.

Carbon dioxide breathed by the mother cat started rhythmical respiratory movements of fetuses normally apneic, but served only to depress further those fetuses which were in a state of partial anoxemia.

Near term, fetuses 62–65 days old executed breathing movements normally *in utero*. Anoxemia and carbon dioxide in excess did not stimulate, but depressed, the respiratory activities of these specimens.

Normally occurring but intermittent rhythms of intrauterine respiratory movements were co-ordinated with phasic uterine contractions. They seemed to result from improved oxygenation of the fetuses at the time of increased uterine tone.

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PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER. II. GENERAL PRINCIPLES OF DEVELOPMENT WITH SPECIAL REFERENCE TO THE AFTER-FEATHER

(Eighteen figures)

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I. INTRODUCTION

FEATHERS represent a special kind of biological system beautifully adapted on account of the relative simplicity and symmetry of structure and pattern to a great variety of biological problems. Their special suitability as test objects for hormones and various physiological conditions and for genetical problems, especially on the borderline between genetics and physiology, is being very generally recognized. Basic to the interpretation of whatever biological problem for which they may furnish material is an understanding of their development. The present paper is, in a sense, a continuation of our interrupted first study in this field published in 1932. The collaborative studies of Juhn and Fraps (1934, 1936), the criticism of Hosker (1936), and our own experience since have resulted in some clarification and modification of views expressed in 1932, as well as additional material, and it seems necessary, therefore, to state

these, though only rather categorically in the present paper, before proceeding to the special consideration of the after-feather. As a matter of fact, a correct understanding of the special problem depends on the more general ones.

The present study is not comparative as between species, although we explicitly recognize the importance of such studies; but we believe that they will proceed more successfully on the basis of a fairly complete analysis in a single form. The study of the regeneration of certain definitive feathers in two breeds of fowl and crosses between them is, in fact, a large undertaking. Our study is limited to them, and in the introductory considerations, to a partial presentation of certain general problems.

II. GROWTH AND MOVEMENT IN THE FEATHER GERM

The feather germ grows in length, *axial growth;* its base (collar) grows somewhat in diameter, *radial growth;* and the barbs, arising in the "ventral" region of the collar, progress in their growth around the periphery of the collar from ventral to dorsal position, *tangential growth or movement.* The foregoing dimensions of growth have reference to the germ as a whole. The barbs and subordinate elements also present their special problems of growth.

A. AXIAL GROWTH

The growing feather presents an apparently straight axis, so long as it is entirely inclosed in its sheath, when it is commonly called the pinfeather. The gentle dorsoventral curvature characteristic of certain contour feathers is not then apparent. From this we conclude that axial growth from the collar is uniform at all transverse levels, or at least very nearly so. Certain important conclusions emerge from this fact, among them the principle that transverse sections parallel to the collar, which is the center of axial growth, intercept points of identical or nearly identical age from the dorsal to the ventral surface.

On the other hand, and without any prejudice to the principle of equal axial growth at all transverse levels, the rate of axial growth is subject to variation in time which has been measured and recorded in the form of curves by Juhn, Faulkner, and Gustavson during formation of the individual feather and in different feather tracts (1931).

The collar is the source of the axial growth, and it is necessary, therefore, to analyze its activities in order to appreciate how the axial growth is initiated and sustained. Figure 1 is a photograph of a frontal (longitudinal) section of the base of a feather germ of 18 days' regeneration from the breast of a Brown Leghorn capon. It shows the regenerating feather in its follicle. Attention is directed at this time to the thick obliquely placed collar surrounding the umbilicus where the central artery enters, hence appearing on both sides, and to the sharp angle that separates it from the forming barbs. In the higher magnification of the right side (cf. Fig. 1) shown in Figure 2, it is seen that the surface of the collar toward the pulp is covered by a high epithelium with tapered cell bases between which other elongated epithelial cells are inserted. Cell division in the collar is limited to such cells, the nuclei of which are arranged in several layers. Immediately next to the barb-forming groove (ramogenous zone, *r.z.*, Fig. 2) these cells contribute to the zone in question. Next to the umbilicus they are continuous with the regeneration cells at the bottom of the follicle. But the great mass of cells of the collar beneath the high epithelium is concerned in axial growth by formation of the sheath of the feather germ. Typical sheath cells, recognizable cytologically, are formed beneath

the dividing cells; they elongate, swing through an angle of about ninety degrees, undergoing keratinization, and form the sheath in the manner indicated in Figure 2.

The details of this interesting process will not be described here. It is sufficient to note in this place how relatively enormous a part of the activities of the collar are employed in the formation of the very dense, highly resistant, and strong sheath. It is from the sheath, in process of growth during the entire regeneration of the feather, that the energy and the mechanical support for continued axial growth are derived. The forming barbs are carried out with it in the manner described in Part C. The shaft has its own basis of growth in the collar, and its rate of growth is conformable to that of the sheath. The pulp is proliferated from the feather papilla (Fig. 1, *P.*) at a rate determined by the general axial growth.

B. RADIAL GROWTH

The increase of diameter of the collar during regeneration (radial growth) is of the order of 50 per cent in breast feathers during regeneration from the ninth to the fortieth day; and considerable reduction occurs in the last stages of growth. It is mentioned only for the sake of consistency because it does not appear to have any very definite relation to the problems under consideration.

C. ORIGIN AND GROWTH OF THE BARBS AND THEIR TANGENTIAL MOVEMENT

We stated in the first paper of this series (Lillie and Juhn, 1932) that two stages may be recognized in the origin of the barbs of the feather. In the first, which we estimated to include about twenty-five apical barbs on each side (in the breast feathers of the Brown Leghorn), the barbs appear to arise practically simultaneously. These barbs are placed vertically in the germ, as shown in Figure 3, Plate I, of our previous paper, thus showing absence of tangential movement as described below. Thereafter, we maintained, the origin of barbs is strictly serial from the ventral (antirhachis) region of the collar, so that each more apical barb of the definitive feather, situated more dorsally in the germ, is slightly older than the next basal. To this principle we still adhere as fundamental. Such variations as will appear beyond are consistent with the conception of ventral origination in the collar, which is a double gradient system as clearly proved by the hormone experiments described in our previous paper.

Our present conceptions demand a broader terminology for the ventral barb-originating region. The term "ventral triangle," which we originally used, will continue to be used in the restricted sense proper to it as descriptive of the arrangement of barbs immediately after their origin with bases divergent and apexes meeting in the mid-ventral line (Fig. 7, *et pas.*). This configuration, however, is due to the occurrence of tangential movements of the bases of the barbs (sec. C, 2). We shall therefore introduce the term "ventral locus" to designate the precise locus of origin of new barbs which is situated in the collar at the base of the ventral triangle when this configuration exists. When tangential movements are active, the ventral locus is highly restricted. Just preceding the formation of the after-feather, and for a short time after, it broadens out, tangential movements cease, and the conformation known as the ventral triangle disappears, to be replaced later by two (Figs. 12–17). Speaking theoretically, the ventral locus is the area between the ventral ends of the lateral collar gradients. The time of the original localization of the ventral locus is not strictly known, but it becomes clearly defined

during the formation of the barbs of the tip of the feather. The term "ventral field" is used for the region in general, of which the ventral locus is the center.

We shall deal first with the movements that affect the disposition and growth of the barbs, and afterwards with their cellular origin.

1. AXIAL GROWTH OF THE GERM IN RELATION TO THE BARBS

The forming barbs are carried passively away from the collar by the axial elongation of the germ, effected as we have seen by the development of the sheath. The fact that they nevertheless maintain contact with the collar is explained by their continuous basal growth at the expense of the cells of the ramogenous zone immediately adjacent to the superior lip of the collar. We shall assume that the relation of the barbs or of any portion of the barbs that is once organized is fixed in relation both to the sheath externally and to the pulp internally; that is, there is no shifting of developing barbs on sheath or pulp. The consequence of these principles is that each formed locus of any barb is carried strictly axially during the entire period of its formation (cf. Fig. 3).

2. TANGENTIAL GROWTH IN RELATION TO THE BARBS

The term "tangential growth" ("tangential movement" of Juhn and Fraps) relates solely to the barbs; it includes the apparent movement of the barb bases from the ventral locus to their junction with the shaft (Fig. 3) and the growth forces responsible for this movement. In our former paper we interpreted this movement as due to a movement of the entire collar directed dorsally from the mid-ventral line and its utilization in formation of the shaft by total concrescence; growth centers for the barbs were presumed to be located in the collar and to be carried along by the moving halves of the collar, which thus dragged the forming bases of the barbs along with them. We can no longer support this interpretation *in toto;* in place of concrescence of the entire halves of the collar to form the shaft, we are concerned with concrescence of derivatives of the collar, which we now regard as itself a fixed system, to form its lateral surfaces only (cf. Fig. 9); and in place of postulating barb centers within the collar we regard the basal growth of the barbs as due to their own intrinsic utilization of material of the ramogenous zone derived from the collar.

The net result of the principles concerning the relations of axial and tangential growth to the formation of the barbs may be expressed in a diagram (Fig. 3).

At any given time, however (cf. Fig. 7 and other photographs of split preparations), a large series of successively formed barbs is present. In such a complement it follows from the foregoing principles and the serial order of origin of barbs from the ventral locus, (*a*) that the length of a barb in the germ as given by the axial position of its apex with reference to any complement of barbs simultaneously present accurately reflects its time of origin, (*b*) that in the absence of tangential movements the orientation of a barb in the germ will be strictly axial or vertical, and conversely, that the obliquity of a barb or of any of its axial parts in the germ is a measure of the rate of tangential motion with respect to axial growth, which may be treated as a constant.

3. BARBS OF IRREGULAR LENGTH

In accordance with the principle of strictly serial order of origin, all the barbs of any simultaneous complement should be graded in order of increasing length from the ventral locus to the shaft along the collar; but this is not always the case. Barbs of irregular

length, longer than those adjacent to them on the shaft side, occasionally occur, and these are more frequent in the later stages of regeneration of the feather during the formation of its fluffy part, and especially during the time of origin of the after-feather.

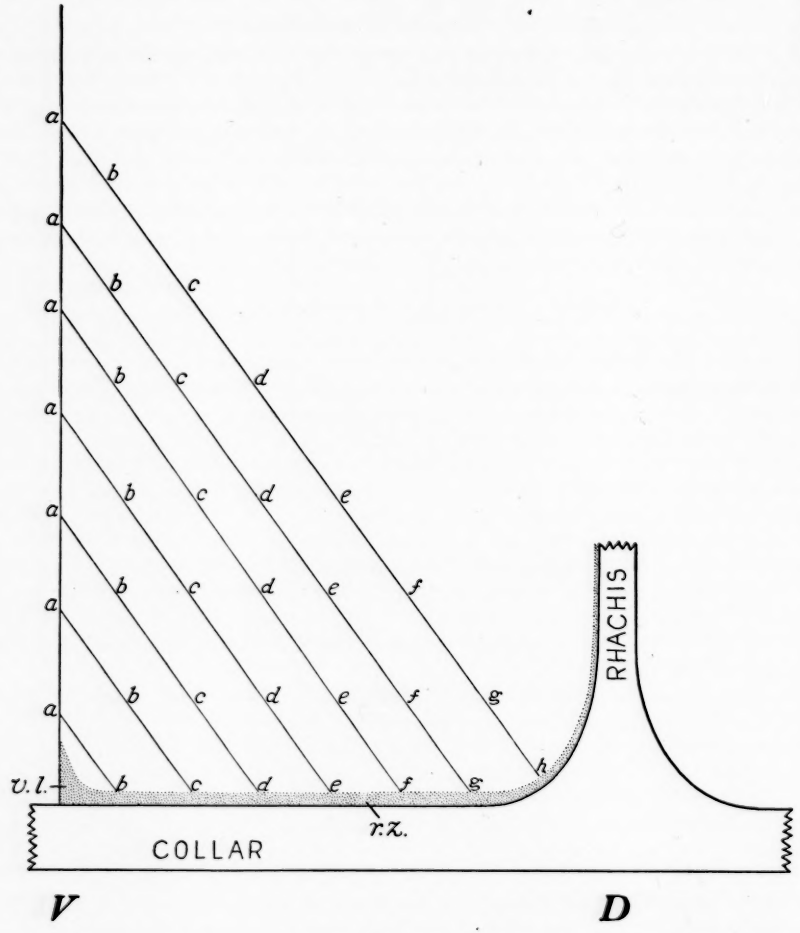

FIG. 3.—Seven successive positions of a single barb during its growth. Successively formed points at the base, *b*, *c*, *d*, *e*, *f*, *g*, and *h* are carried vertically at an equal rate, owing to uniform axial growth at each of these transverse levels. Owing to tangential movement, the successively formed basal points move toward the rhachis. The oblique orientation of the barb is thus due to a parallelogram of axial and tangential forces, which in the diagram are assumed, as one limiting case, to preserve a constant ratio during the entire formation of the barb. The apex of the barb, *a*, maintains its position at the mid-ventral line. *D* = dorsal; *V* = ventral; *r.z.* = ramogenous zone; *v.l.* = ventral locus. ×30.

The mode of origin of such barbs is as follows: Inasmuch as tangential movements are away from the ventral locus on both sides of the collar, there is a dead center at the ventral locus itself. Any barb that forms in this dead center is carried vertically by the axial growth of the germ (Fig. 4). In the meantime, other barbs are formed that participate in the tangential movement from the start. Subsequently, the barb in the dead center is caught up by the tangential movement, and therefore takes a place more ventral than barbs of later origin which have participated in the tangential movements from their start, although it is longer than they. Such barbs, while in the germ, always betray their origin by a relatively abrupt angle between the vertical part remaining in the dead center and the oblique part formed after participation in the tangential movements (Figs. 5, 6, *et al.*).

It is evident that the probability of formation of barbs of irregular length will depend upon the extent of the dead center corresponding to the ventral locus. During the formation of the vane of the feather the ventral locus is particularly restricted, but at the time of formation of the after-feather it becomes much broader, a condition that will be fully described beyond. Barbs of irregular length are very rare in the vane of the emerged feather, but are the rule rather than the exception in the apical part of the after-feather.

Hosker (1936, pp. 180–81) interprets the occasional occurrence of barbs of extra length on one side of the vane as indicating that "the ventral point is not fixed, but in this region of plasmatic growth, a barb which lay nearer to the left side of the feather germ might grow towards the right side of the rhachis." Such a denial of principle is fortunately unnecessary. It is impossible to visualize how a single barb can step out of the ranks at one place and rejoin them at another; but if a transference of the ventral locus from one position to another is meant, whole series of barbs, and never single barbs, must be involved.

4. CELLULAR BASIS OF ORIGIN AND GROWTH OF THE BARBS

The barbs originate and grow from a narrow zone of loosely arranged cells immediately adjacent and apical to the collar, which we shall call the "ramogenous zone." The rhachis interrupts this zone dorsally, but it is otherwise a continuous narrow ring (Fig. 3). It is located in the angle formed by the collar and the vertical walls of the feather germ (Fig. 2). The cells composing it appear to be derived by continuous proliferation of the adjacent (apical) margin of the collar; they are smaller than those of the collar epithelium, discrete and rounded; intercellular spaces produce the appearance of a network of strands; the epithelial surface next the pulp is here devoid of a basement membrane. The nuclei of the cells form about eight to ten layers, and karyokinesis may occur at any level. Transverse sections show well the reticular character of the ramogenous zone (Fig. 8). The barb septa disappear in this zone, which is entirely unsegmented, and all stages in the building-up of the bases of the barbs from the cells of the ramogenous zone can readily be seen. At some time we propose to publish a more detailed account of the foregoing interesting relations.

The ramogenous zone is the only part of the epithelial constituents of the feather germ which is not structurally coherent. It alone is structurally adapted to the execution of the tangential displacement of the bases of the barbs.

5. THE ORIGIN OF BARB APEXES

The origin of new barbs, i.e., of barb apexes, like their growth, is always from the ramogenous zone, but it is confined to the ventral locus, the extent of which is subject to

some variation as we have seen (cf. p. 436). This locus is situated between the two gradients of the collar, and it is a tempting thought that its extent is a function of the steepness of the gradients. During the greater part of the growth of the feather it is surmounted by a triangular organization (Fig. 6) of cells of the ramogenous zone with the apex directed distally. The base rests on the collar, and the sides are split off as new barb apexes.[1] The latter thus have a considerable angle of divergence which is preserved as the combined forces of axial and tangential growth transport them in the manner already described (Fig. 3). In the region of the ventral locus the process of forming new barb apexes is then repeated. Right and left barb apexes need not be formed simultaneously. These processes have a practically invariable form, except during the formation of the apex of the vane and of the after-feather.

The barb apex once formed in the manner just described appears to act as a center of organization of the cells of the ramogenous zone; as it moves toward the shaft it continues to utilize cells of the ramogenous zone, thus availing itself of cells of all levels of the physiological gradient. The point at which growth ceases and its union with the shaft is complete is rather difficult to identify; cells of the ramogenous zone occurring between the bases of the growing barbs are carried with them on the lateral surfaces of the growing shaft. They are responsible for the origin of barbules arising directly from the shaft.

6. ANALYSIS OF TANGENTIAL MOVEMENT

Axial growth and movement is readily understood in principle on the basis of cell divisions and movements. It is not so with tangential movement, for there is no distribution of cell divisions and no evidence of streaming of cells in the direction of the movement to account for it. Various views are theoretically possible.

a) It might be supposed that an original oblique orientation of the part of the barb first formed persists and controls the direction of organization of all its later parts. This would approximate Hosker's interpretation, as I understand it. (Hosker, 1936, p. 176). This, however, would not hold for the apical barbs which are at first vertical (Lillie and Juhn, 1932), nor would it hold for the barbs forming the apex of the after-feather which are also vertical (see p. 445), and it would not account for definite changes in orientation during development, which are especially noticeable in the formation of barbs of exceptional length (p. 437). An essential part of the explanation of tangential movement must be found in conditions external to the forming barb as such.

b) A second theory might be based upon a reaction of the forming barb bases to the physiological gradients, which are evidenced in general by a high threshold of reaction to hormones next to the ventral locus corresponding to the barb apexes and a threshold diminishing in a dorsal direction along the collar to junction with the shaft. The theory would be in general terms that tangential displacement follows the gradient, and that its rate and hence the obliquity of the forming portion of any barb is proportional to the steepness of the gradient. This would be consistent with the vertical orientation of barbs in the "dead center," i.e., the place where the opposite collar gradients are in equilibrium. It is believed that a consistent theory might be formed upon this basis.

c) A third theory might be based on the postulate that successive formations of new barbs in the ventral locus act mechanically as a *vis a tergo* pushing the barb bases dor-

[1] Many feather germs, notably of the primary flight feathers, have strikingly asymmetrical ventral triangles, though symmetrical sides are more usual.

sally. This is the most obvious interpretation. However, the force seems inadequate to control the entire complement of barbs existing at any one time, unless it is supposed that such a purely mechanical factor is supplemented by a physiological relationship, such as is suggested in the second theory.

It is to be noted, with reference to apical barbs of both main and after-feathers, that the origin of tangential movements is synchronous with the origin of the shaft (cf. Figs. 12, 13, 14). It is also obvious that continuous axial growth of the shaft is a necessary condition for continuation of tangential movements of the forming barbs. These processes have a certain necessary association and correlation. The growth of the shaft has thus also to be taken into account in the analysis of tangential displacement.

D. THE FORMATION OF THE SHAFT AND THE ROLE OF CONCRESCENCE

In our previous paper (Lillie and Juhn, 1932) we ascribed the origin of the shaft simply to concrescence of the lateral halves of the collar. Renewed study has shown that the matter is not so simple; indeed, four longitudinal components may be distinguished throughout the extent of the shaft, a ventral and a dorsal forming the central axis, and two lateral components which fuse with the central axis.

The origin of the apex both of the shaft and also of the aftershaft is always indicated first by a properly located projection from the collar (Figs. 13 and 14, and Fig. 3 of our 1932 paper). Before this time the barbs are vertical with reference to the germ (cf. Figs. 12 and 13), and the bases are not in any way fused. As the shaft primordium elongates, it carries the apical barbs at first, and later, successive barbs with it. The bases of these barbs and the ramogenous material between them contribute to the lateral surfaces of the shaft primordium, as is very clearly demonstrated by the barbule-forming cells incorporated in this material.

The median part of the shaft which thus arises independently of the barbs has ventral and dorsal components of separate origin. The ventral component is derived from the collar epithelium which extends also in an active state along the ventral surface of the shaft for a certain distance until the smooth keratinized ventral surface is differentiated. In its actively dividing region it produces the cells that differentiate into the medullary cells of the shaft. But the keratin of the dorsal surface, which is separated from that of the ventral surface by the medulla and the lateral components, is derived from cells of independent origin placed between the medulla of the shaft and the feather sheath. Beyond saying that the cells in question are closely associated with the inner layer of the feather sheath, we do not wish to commit ourselves as to their origin.

The median part of the shaft arises from a spreading base in the collar (Fig. 7), which is also characterized by a much less massive development than the remainder of the collar (Fig. 8). The spread of the base indicates a central concentration of material in the formation of the shaft itself. The disposition and orientation of the melanophores which lie beneath the collar epithelium also indicate a lateral source of origin for those which enter the shaft (Figs. 7 and 17).

We conclude, then, that a process of concrescence is concerned in the formation of the lateral surfaces of the shaft, and that there is some concentration of material toward the center in the formation of the median part of the shaft. In any event, the median part of the shaft is of entirely different embryological origin from the barbs.

The diagram (Fig. 9) summarizes certain features of the foregoing description.

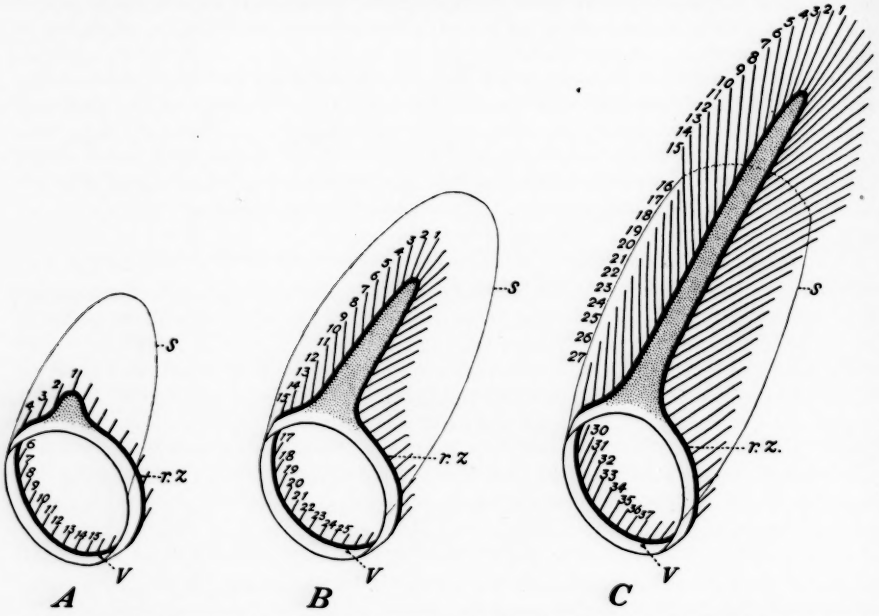

FIG. 9.—The diagrams represent three stages in the development of the feather. The two halves arise from a ring of embryonic cells, the collar, sunk in the feather follicle, and surrounding the base of the feather germ; the ridges or rami after the first complement (diagram *A*) arise in the ventral locus (*V*) grow at their bases from cells of the ramogenous zone (*r.z.*) adjacent to the apical margin of the collar, and simultaneously move tangentially on each side to the rhachis, carrying with them cells of the ramogenous zone to form the lateral surfaces of the rhachis. As the axis of the rhachis (stippled) grows out from the dorsal region of the collar it carries the completed barbs with it. As a result of the tangential movements of the bases of the barbs, space is constantly provided at the ventral locus for origin of new barbs which arise serially on each side of the ventral locus (diagrams *B* and *C*, 16–37). The sheath (*s*) arising from the proximal lip of the collar (umbilicus) is ruptured apically after a certain stage (*C*).

At any given time the collar is beset with a series of forming barbs on each side which decrease in age from the rhachis to the ventral locus, and in state of development from the completely formed barbs, nos. 1–25 (diagram *C*) to mere apical rudiments ventrally, nos. 35, 36, 37 (diagram *C*).

The order of formation of the shaft is apicobasal, and the order of age of the barbs the same. Similarly, in each barb the apex at the margin of the feather is the first formed and the central end attached to the shaft last. Thus there are two time gradients in each feather from apex to base along the shaft and from margin to center along the barbs. (Modified from Juhn, Fig. 43, ch. xiii, *Sex and Internal Secretions*, Baltimore, 1932.)

E. SUMMARY

1. The principle changes in our 1932 account which we have made are as follows:

a) Origin of the shaft: The principle of concrescence is limited to the formation of the lateral surfaces of the shaft.

b) Origin of the barbs: We have given up the principle of individual growth centers situated in the collar.

c) The explanation of the physiological gradient along the axes of the barbs evidenced by a rising threshold of reaction to thyroxin and female hormone from base to apex, or in the germ from dorsal to ventral surfaces, which was given in our 1932 paper, also requires modification. We are now uncertain whether the descending physiological gradient is correlated with a descending rate of growth of the barbs from apex to base. In any event, the gradient of growth-rate must be much less steep than was postulated.

2. The main additional principles that we have stated in the present paper are as follows:

a) The collar as such is a *fixed* double gradient system, the intensity of which is subject to quantitative variations during the development of the feather.

b) Axial growth of the germ as a whole is approximately uniform at transverse levels; however, it may vary at different axial levels, i.e., in time.

c) The sheath of the feather germ developing from the collar is the principal mechanical supporting element of axial growth.

d) The ventral locus situated at the "dead center" between the two gradients is subject to the stated variations in extent, relating especially to the origin of the after-feather (*q.v.*).

e) The apical segments of barbs act as organizers of ramogenous material arising from the collar; this conception replaces the theory of growth centers in the collar.

III. THE AFTER-FEATHER

A. INTRODUCTION

The general principles of this section were stated by us in a preliminary paper (Lillie and Juhn, 1937; see also Lillie, 1937).

The after-feather of fowl corresponds in length to a somewhat variable portion of the fluffy region of the contour feathers, never extending into the region of the vane. It emerges, as is well known, from the superior umbilicus, and its ventral surface is applied to the ventral surface of the main feather. It is well developed in connection with unspecialized contour feathers but is practically absent, or represented at most by a few barbs, in the flight and tail feathers. In the neck hackles it is absent or rudimentary anteriorly, but shows a gradually increasing development to a typical condition next to the cape and back. Its distribution in birds in general is given by Chandler (1916). Our account of its development will be confined to the breast and saddle feathers in fowl.

In these tracts it comprises the same parts and elements as the corresponding part of the main feather. Its parts measured from apex to base are synchronous in origin with the identical transverse levels of the main feather and partake of the same general fluffy structural characteristics. It is not as broad as the main feather, and its length relative to the main feather varies considerably, apparently in relation to its position in the feather tract or field. Reference to Figures 10, 11, and 18 will be sufficient for purposes of general orientation.

The study of the development is based upon over five hundred total preparations and a large number of serial sections, including all important stages. The early stages will be classified by the total number of barbs of the after-feather formed or in process of formation. After the formation of the hyporhachis, which occurs usually at or about the

stage of six barbs, they are, of course, divided into right and left series approximately equal in number on the two sides.

The sex of the bird, the tract concerned, and position within the tract are intrinsic variables affecting the time of beginning of the after-feather reckoned from time of plucking. The following data are taken from accurately timed, systematic pluckings of breast and saddle feathers in a male and female Barred Rock respectively, involving over one hundred follicles: in the female the after-feather in the breast begins to form about the thirtieth day after plucking, when the length of the regenerating feather is from 4.6 to 5 cm.; in the male, formation begins a little later than in the female, at about thirty-five days. The saddle of the female is quite similar to the breast in these respects; but in the male the time of origin is much longer, about forty-three days. In the breast of both sexes the lateral feathers in positions 7, 8, and 9 (see Holmes, 1935) are later in beginning than those in positions 1–6; number 9 indeed may have only one afterbarb at a time when the average in numbers 1–6 is 42.

These observations suggest problems of considerable interest, especially in relation to field functions. However, they are mentioned here merely as a guide to the description of stages that follows. The foregoing data for the Barred Rock are controlled to a certain extent by other less complete series. Season of the year, condition of the bird, and repetition of pluckings affect the onset of regeneration, and hence the age reckoned from plucking.

The general plan of development of the after-feather may be readily understood by examination of total preparations. These are made by splitting the cylinder of epithelial cells surrounding the pulp on one side from the umbilicus longitudinally, spreading it out flat, pulp side out, and fixing it under pressure between two glass slides; the preparation is then dehydrated, cleared, and mounted without staining. Such a preparation affords a view of the interior of the feather germ and displays an astonishing amount of detail. The preferred lines of incision are near the dorsal surface, midway laterally, or near the ventral surface. The last is not suitable for the study of the after-feather. The constriction of the umbilicus (see Fig. 1) necessarily submits the region of the collar to considerable strain in this method of preparation, and it must be realized that the delicate bases of the barbs may thus be subject to some distortion. Allowance must be made for this in using such preparations, but it does not affect the subject under consideration in any essential way.

Figure 7 exhibits the basal portion of an entire lateral split preparation made about one week before the time of origin of the afterbarbs. The photograph is useful for general orientation, and attention should be directed especially to the conformation of the ventral triangle.

B. DEVELOPMENT

1. IRREGULARITIES IN THE VENTRAL LOCUS

Stages immediately preceding the actual origin of the after-feather are characterized by a greater frequency of barbs of irregular length. It will be remembered that these arise from the apex of the ventral triangle, remain in the dead center, and thus grow vertically for a variable period of time before being taken into the tangential stream. They are accordingly characterized by greater length than neighboring more dorsal barbs, and also by a rather abrupt angle between the vertical apical part and the oblique part formed after entering the tangential stream (cf. Figs. 4, 5, 6, and 12).

At this time also, and during early stages of the after-feather, other irregularities in

barb formation appear quite commonly in the ventral region; barb apexes may be completely detached (see Fig. 6), bases of barbs may appear constricted, or two apexes may have a common base. Such abnormalities are frequent enough to indicate a considerable disturbance of the ventral region. This instability persists for a certain period during the early development of the after-feather, but disappears later.

2. THE ORIGIN OF THE FIRST AFTERBARBS

The formation of the after-feather is initiated by the origin of a series of vertical barbs from a broadened ventral locus (Fig. 12). In accordance with our interpretation of vertical barbs (p. 439), tangential movements have now ceased in this region, while axial growth continues. Also according to our interpretation of the relation of axial growth to length of barbs, the length of a barb is a measure of its age with reference to its neighbors. Accordingly it will be observed that the first vertical barbs do not arise in a regular sequence like their predecessors and successors. For instance, in Figure 12 the three afterbarbs are of increasing age from left to right. This irregularity in origin of the first afterbarbs is reflected in the apex of the after-feather.

It is interesting to recall that the apical barbs of the main feather arise also in a vertical position before any sign of the shaft appears, after which tangential movements begin and the bases become oblique as they join the shaft (Lillie and Juhn, 1932).

3. ORIGIN OF BILATERAL SYMMETRY OF THE AFTER-FEATHER

The origin of bilateral symmetry of the after-feather is associated with the appearance of the hyporhachis and the distinct delimitation of two ventral loci.

a) The two ventral loci are derived from the lateral margins of the broad ventral locus of the preceding stage. This may be interpreted as a process of division or budding of the original single ventral locus; its activity becomes concentrated at its margins which have been separated by the vertical afterbarbs. Each new locus has now the same bilateral activity as the original one, forming barbs right and left of its center (cf. Figs. 13–17). In terms of the gradient theory, there are now four gradients in place of two, directed specifically from each center toward the rhachis of the main feather and toward the hyporhachis. Each locus contributes to the right side of the main feather and the left side of the after-feather, or vice versa. The consequences of this will be considered later.

b) Bilateral symmetry of the after-feather itself is established by the origin of the hyporhachis in the approximate center of the vertical afterbarbs, when about six are present. In the entire preparation (Fig. 13) it appears as an unpigmented moundlike projection of the collar into the bases of the barbs, whose pigmented bases are entirely distinct from it. As it rises it carries the afterbarbs with it. It is plain, and must be emphasized, that the hyporhachis is an independent formation derived from the collar, and that the attachment of the barbs is secondary, as is also their contribution and that of the ramogenous material to its lateral surfaces.

As soon as the hyporhachis is established, tangential movements of the bases of the barbs toward it begin, clearly indicated by their oblique disposition. This is already to be seen in one of the barbs of Figure 13. In the stage of eighteen afterbarbs shown in Figure 14, tangential movement is already actively under way.

4. LATER DEVELOPMENT OF THE AFTER-FEATHER

Later stages in the development of the after-feather are shown in Figures 15, 16, and 17. In the last, which shows only its basal portion, the after-feather is near completion.

Study of these figures and of their captions will serve better than any detailed description in the text for a survey of the progress of events. The relations of the two ventral loci and their triangles to the main feather and after-feather, the elongation and attenuation of the hyporhachis, and the steady addition of new barbs to both feathers are especially to be noted. Throughout these stages tangential movement of barbs, reflected in the obliquity of their bases, is active in both directions from each ventral locus. After the period of irregularity at the time of origin of the first barbs of the after-feather, the ventral loci have settled down to machine-like regularity.

5. DISCUSSION

a) *The conception of collar gradients.*—The conception of gradients in the collar is based upon our experimental determination (1932) that the threshold of reaction to the female hormone or thyroxin in breast and saddle feathers rises in the germ from the dorsal (rhachial) extremity of the collar to the ventral locus, or in the case of the barbs from base to apex. There are thus two gradients corresponding to the two halves of the vane of the feather descending from the sides of the ventral locus in the germ to the sides of the rhachis.

The ventral locus determines the rate of origin of barbs; it thus asserts a kind of dominance over the entire process of feather formation. We shall present reasons below for believing that its dominance expresses itself also in special ways.

b) *Twinning in the origin of the after-feather.*—The two ventral loci associated with the development of the after-feather are derived from the material of one, the original ventral locus. The new ventral loci inherit, so to speak, an established gradient in the collar on the rhachis side of each and are related in similar manner to new gradients on the hyporhachis side of each. The twinning is thus obviously not a division of the whole germ system. But the results, described below, speak for a dominance of the whole system by the new loci.

The twin triangles are much closer to the hyporhachis than to the rhachis, especially in early stages of the after-feather (Fig. 15); and even in late stages (Fig. 17) they do not become equidistant. In the first case, the difference is of the order of eight to one, and in the latter more than two to one. A barb destined for the apical portion of the after-feather thus undergoes only one-eighth as much tangential movement during its growth as one destined for the main feather, but it attains from one-third to one-half its length (Fig. 11). On the principle of equal axial growth at all transverse levels of the germ, i.e., equal increments of length in equal periods of time, it must follow that tangential movement is slower on the hyporhachis side of the ventral loci. An interesting correlation of this will appear later in comparison of the barbs of the main feather and the after-feather.

In accordance with our previous interpretation, each of the new ventral loci must be regarded as the high point of a double gradient system which terminates in hyporhachis and rhachis, respectively. They thus enjoy a certain degree of independence, being blocked off from one another by the rhachis on one side and the hyporhachis on the other.

In the Barred Rock fowl (Montalenti, 1934) the initiation of the black bars begins at the ventral triangle and progresses toward the rhachis. When it is reached the white bar begins to form. Black and white phases of activity are not necessarily quite synchronous on the two sides of the vane. It thus frequently happens that in preparations one side next to the ventral triangle may be in the black phase and the other side in the white phase (cf. Fig. 5). The two sides are "out of step," so to speak. Similarly, the two ven-

tral triangles of the after-feather stage may be out of step. In such a case the one in the black phase will be associated with asymmetrical black spots placed on morphologically opposite sides of the main feather and after-feather, but superimposed, owing to the face-to-face relationship (see p. 443).

This phenomenon makes it necessary to postulate dominance by the ventral locus in the process of pigment formation (development of melanophores), for the only thing that opposite halves of the vanes of the twin feathers have in common is precisely the common ventral locus. This conclusion is of interest not only in a physiological but also in a genetic sense, because Montalenti has shown that the rhythmicity in barring is a function of the genes, which are therefore shown in their turn to be controlled as to time of action by a physiological system, described by us in terms of a gradient hypothesis.

C. PATTERNS IN THE DEFINITIVE AFTER-FEATHER AND THE MAIN FEATHER

In most breeds of fowl, pigment patterns are not found in the after-feather nor in the corresponding part of the main feather. The Barred Rock is an exception, however, for the barred pattern extends in the best examples to the base (Fig. 10). This enables comparison of pattern in the twin feathers. The bars correspond in the two so closely that they very nearly superimpose in the natural position of the feather; the black and white phases in development have been synchronous in the twin feathers, or nearly so. Moreover, defects in barring are repeated in the twin feathers, frequently at least. As an example, the second black bar of the after-feather in Figure 10 is defective on the observer's left in both; this is, however, actually the right side of one and the left side of the other. In the few cases in which barring is absent, it is so in both partners, and the ground tone, white to dark grey, is the same in both.

But the most remarkable examples of twin asymmetry and its reversal of side in our material are found in the hybrids between Brown Leghorn and Barred Rock. Here they are also very numerous. In these hybrids typical barring never extends into the fluffy region of the main feather or, *a fortiori*, into the region of the after-feather, though parti-colored patterns may occur. The superimposed regions of the partners are always the same (Figs. 11 and 18), thus all white or all gray or black, or if parti-colored, the resulting patterns are the same in both. There are various types of parti-colored patterns. For instance, a black tip of an after-feather otherwise white corresponds to the level of the main feather where black passes into white (Fig. 18*C*). If a black spot is confined to one side of the after-feather it corresponds accurately in position and form to a black spot of the main feather, though on its morphologically opposite side. The figures and their descriptions will give a better account than much writing in the text.

But, like all other twin characters, there is never complete identity of pattern. The special interest of these twin feathers is that their development explains so many features not only of resemblance but also of difference.

a) Axial level resemblances are explained by the principle of equal axial growth of the germ.

b) The barbs of the main feather are longer than those of the after-feather, for they take more time to complete their tangential movements and have longer time in which to grow. It is of course the basal part of the barb of the main feather that is lacking in the after-feather.

The barb of the main feather in the region of the after-feather consists of two quite sharply distinct parts: a short basal region in which the barb proper is flattened in an

apicobasal direction, thus presenting the flat surfaces toward its neighbors, and a much longer cylindrical part. The transition between the two is quite abrupt. The basal part possesses short stout barbules, and the distal part extremely long (about 6 mm.) hair-like barbules (cilia). In the after-feather the barbs resemble only the long, distal part of the barb of the main feather, and the barbules are also similar. The homologue of the basal part of the barb of the main feather is completely lacking in the case of the after-feather. The obvious explanation is that the growth of the afterbarb ceases too early for the development of this homologue. An interesting corollary of this is that the barbules situated on the sides of the shaft between barb attachments are short and stout in the case of the main feather, and long and filiform (cilia) on the hyporhachis. As a result of this situation, the pigment spots described are narrower on the after-feather than on the main feather and correspond to the marginal zone of the main feather.

c) The reversal of "sidedness" is a necessary result of the fact that when either ventral locus is in a different phase from the other, as in all the asymmetries, its effect is on adjacent sides of the twins which are morphologically opposite.

d) Two or three of the apical barbs of the after-feather have, strictly speaking, no homologues in the main feather. Homologous barbs cannot, in fact, arise until the two new ventral loci are definitively established. These barbs have thus a somewhat special character explained by their developmental history.

There are thus reasons for lack of complete identity of twin characters which are intrinsic and need not be referred to any environmental difference. The significant aspect is that the causes for the dissimilarities mentioned are developmental, not genetic, and that they can be identified.

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PLATE I

FIG. 1.—Feather germ of Brown Leghorn capon from the posterior part of the breast; 18 days' regeneration. *Frontal* longitudinal section in follicle. *A* = central artery of pulp; *C* = collar; *d* = derma; *Ep* = epidermis of skin; *F* = cavity of follicle; *P* = pulp; *Pa* = papilla; *r.z.* = ramogenous zone; *S* = sheath of feather germ. ×ca 22.

FIG. 2.—Collar and adjacent region from right side of Fig. 1. *r.c.* = regeneration cells; note the connection of these follicular epidermal cells with the collar; they represent a special proliferation of the follicular epidermis from which in conjunction with the papilla the next regeneration will take place. When a feather is forcibly plucked it carries with it all the collar and some of the regeneration cells; the pulp is removed *in toto*, but the papilla remains in place. *B'bl.* = barbule cells; *ep.c.* = proliferating epithelium of collar; *r.z.* = ramogenous zone. ×*ca.* 184.

PLATE II

FIG. 4.—Ventral field of a split preparation (see p. 444) of a Barred Rock female from the left anterior breast (feather *l.b.a.*5) to show a central vertical barb in an early stage (cf. p. 439); 24 days' regeneration. *V* = ventral. ×50.

FIG. 5.—Ventral field of a split preparation of a Barred Rock female from the left anterior breast (feather *l.b.a.*9); the left side is in the black phase, and the right side in the white phase of barring. The third barb on the left consists of a vertical apical portion and an oblique basal portion with an abrupt angle between. To illustrate origin of barbs of irregular length see p. 437; 24 days' regeneration. ×50.

PLATE III

FIG. 6.—Ventral field, showing triangle and precise location of ventral locus, from Barred Rock male saddle (feather *l.s.c.*3). Stage just before beginning of after-feather. Barbs of irregular length, right and left; also other irregularities (cf. p. 444). ×*ca.* 100.

FIG. 8.—Transverse section of collar at stage of five afterbarbs (cf. Fig. 12). At this level the main mass of the collar is composed largely of early sheath cells. The feather germ from which this preparation was made was plucked, and the pulp was torn away from the collar on the right side. The section passes through the broad base of the rhachis (*Rh*) (cf. Fig. 7), which interrupts the thickened welt of the collar near its apical margin. A portion of the ramogenous zone appears on each side of the rhachis region (*r.z.*); in more apical sections it can be followed all the way around except in the region of the shaft (cf. p. 439). *A* = central artery; *V* = ventral.

PLATE IV

FIG. 7.—Entire width of base of total preparation split laterally to show relations of ventral field and rhachis. Hybrid capon (Barred Rock × Brown Leghorn); about one week before origin of after-feather; breast (feather *r.b.a.*9); *V* = ventral; *D* = Dorsal. ×30.

PLATE V

FIG. 10.—Barred Rock male: *A*. From left back, row *c;* ventral view with after-feather displaced; *B*. From left breast with after-feather similarly displaced.

FIG. 11.—Hybrid capon (Barred Rock × Brown Leghorn). Ventral view of base of feather with after-feather turned back. *A*. From right saddle, row *d;* main and after-feather all white; *B*. From right saddle, row *c;* main and after-feather with similar black spots; on the right side in the main feather and on the left side in the after-feather, the ventral surface being up in both.

PLATE VI

FIG. 12.—Ventral field in total preparation, stage of three afterbarbs (cf. p. 445); no hyporhachis. Barred Rock female, saddle (feather *l.s.d.*4); 40 days' regeneration. *A'f* = after-feather; first three barbs. ×85.

PLATE VII

FIG. 13.—Ventral field in total preparation. Usual symmetrical arrangement of six afterbarbs (*A'f*) with beginning stage of hyporhachis in the center, appearing as a low rounded projection from the collar. Barred Rock female saddle (feather *r.s.d.*2); 40 days' regeneration. ×85.

PLATE VIII

FIG. 14.—Ventral field in total preparation. After-feather (*A'f*) with nine afterbarbs on each side, and moundlike hyporhachis. The two ventral triangles (*V'*), right and left of the after-feather, are well differentiated. Note the apparent fusion of the two central afterbarbs at the apex of the hyporhachis, and also the irregular lengths of afterbarbs on the right. Barred Rock female saddle (feather *r.s.d.*4); 40 days' regeneration. ×*ca.* 73.

PLATE IX

FIG. 15.—Entire width of base of total preparation, lateral split, to show relation of after-feather (*A'f*) and hyporhachis to main feather and rhachis. About ten afterbarbs on each side. Note arrangement of chromatophores at spreading base of rhachis. Hybrid capon (Barred Rock×Brown Leghorn) breast (feather *r.b.d.*6); about 40 days' regeneration. *V'*, *V'*=the two ventral triangles. ×*ca.* 24.

PLATE X

FIG. 16.—Ventral field in total preparation. After-feather (*A'f*) with about eighteen afterbarbs on each side. Note especially further elongation and narrowing of hyporhachis. Barred Rock female saddle (feather *l.s.f.*1); 37 days' regeneration. *V'*, *V'*=the two ventral triangles. ×*ca.* 60.

PLATE XI

FIG. 17.—Entire width of base of total preparation split close to rhachis on left to show relations of after-feather (*A'f*) and main feather. More advanced stage of after-feather than preceding figures. Note relations of chromatophores in the collar to those in the rhachis. Barred Rock female saddle (feather *l.s.e.*4); 37 days' regeneration. *V'*, *V'*=the two ventral triangles. ×30.

PLATE XII

FIG. 18.—Saddle feathers of hybrid capons (Barred Rock×Brown Leghorn), ventral views with after-feather turned back. Identical patterns of after-feather and main feather with reversal of side. *A*. Capon 229 saddle (feather *r.s.c.*3), vane pure Barred Rock type. *B*. Capon 198 saddle (feather *l.s.a.*1), vane pure Leghorn type. *C*. Capon 198 saddle (feather *r.s.a.*3), apical half of vane Barred Rock type, basal half Leghorn type.

FIG. 1

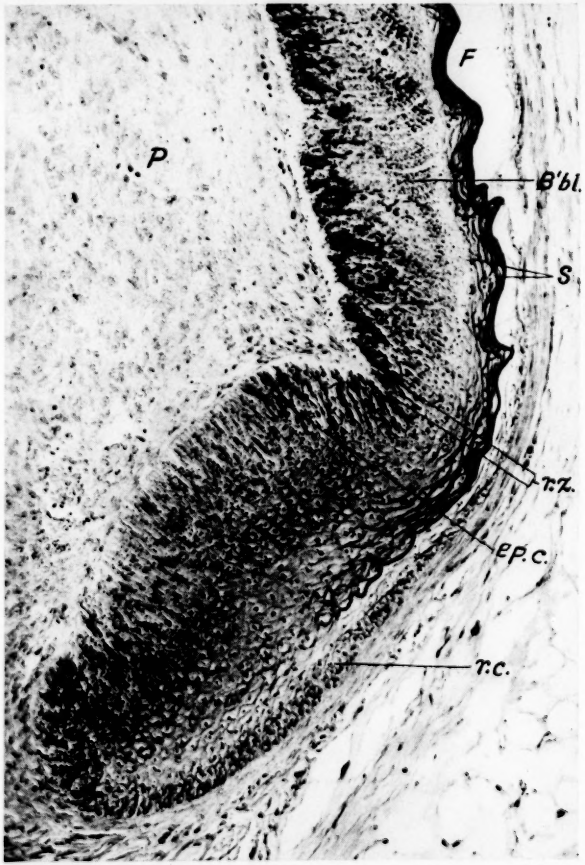

FIG. 2

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FIG 4

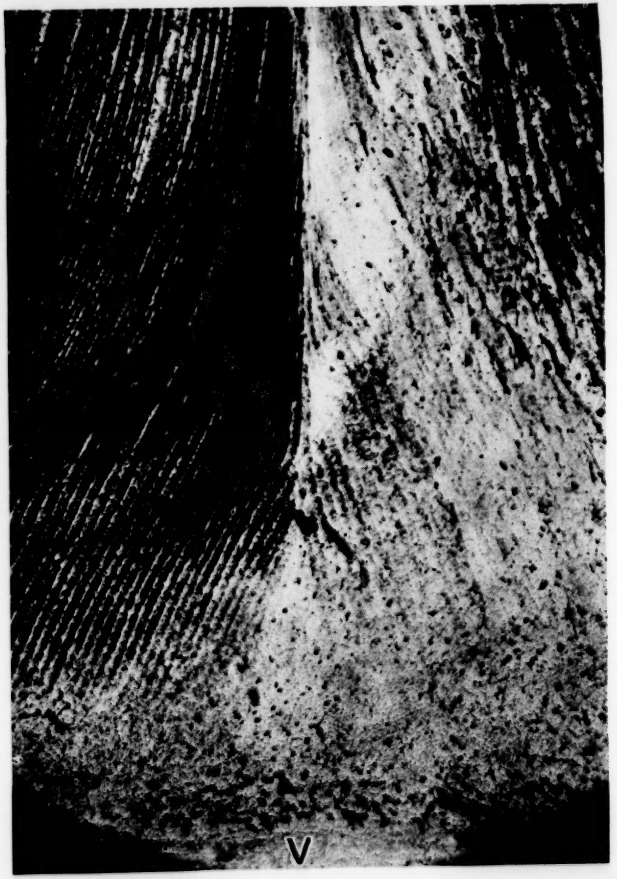

FIG. 5

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PLATE III

FIG. 6

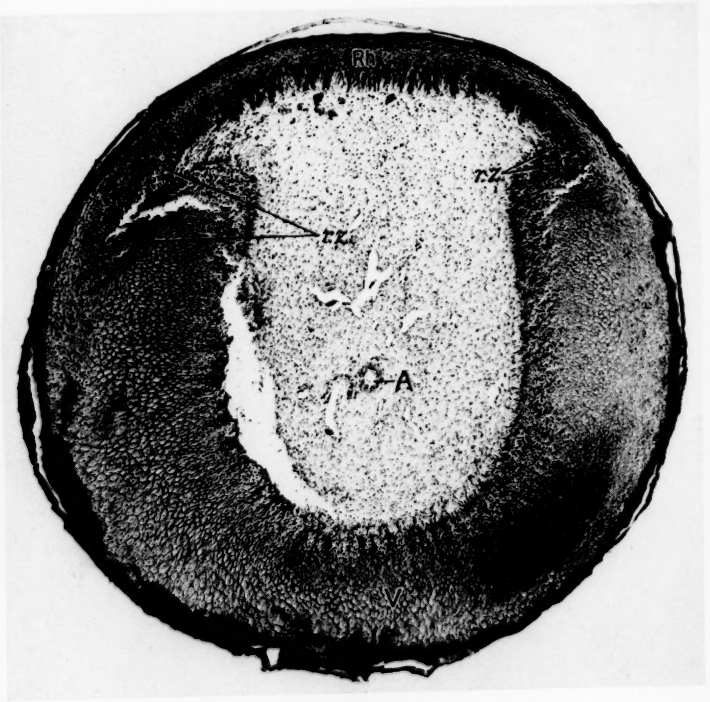

FIG. 8

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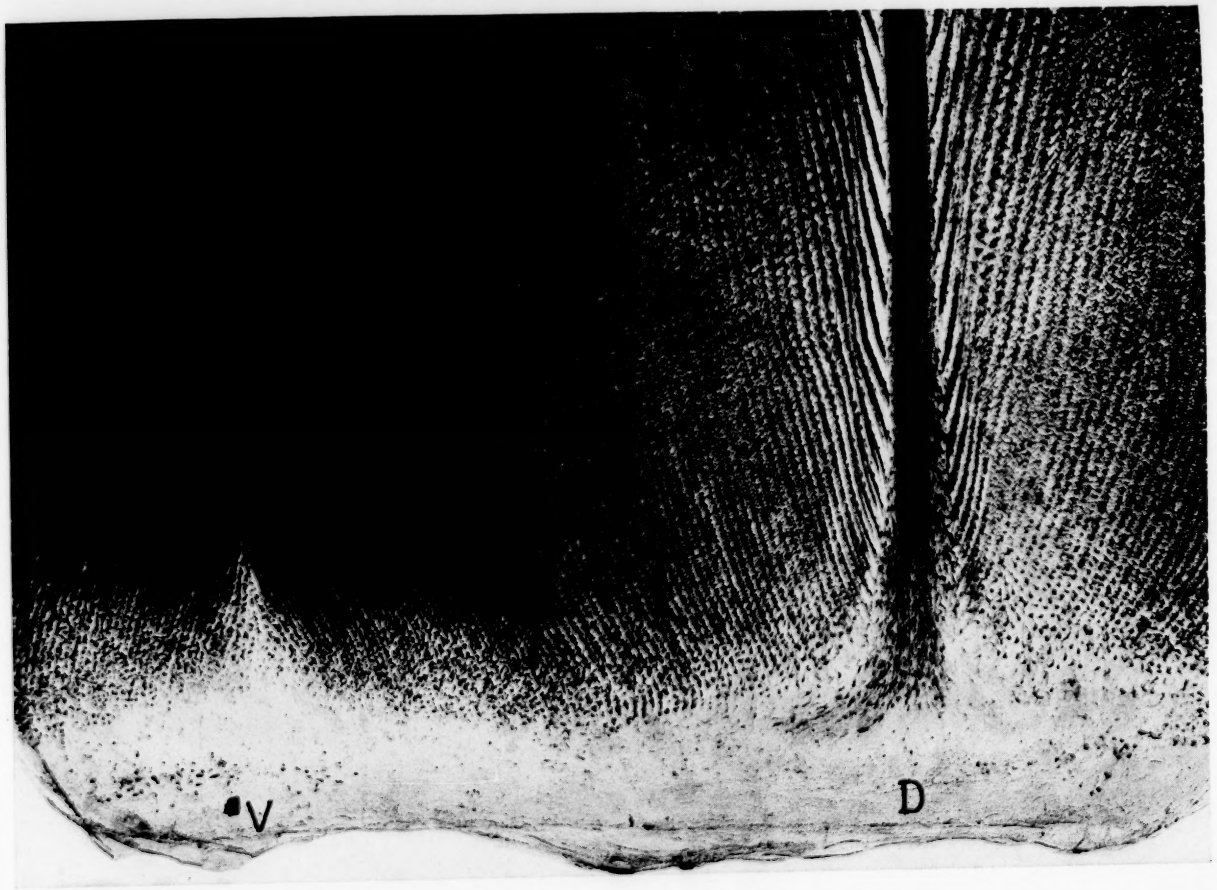

FIG. 7

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PLATE V

FIG. 10

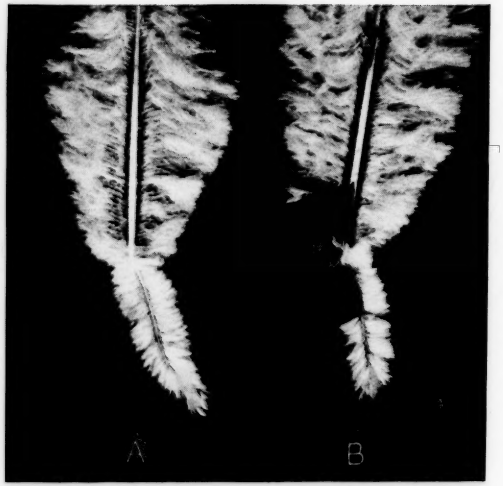

FIG. 11

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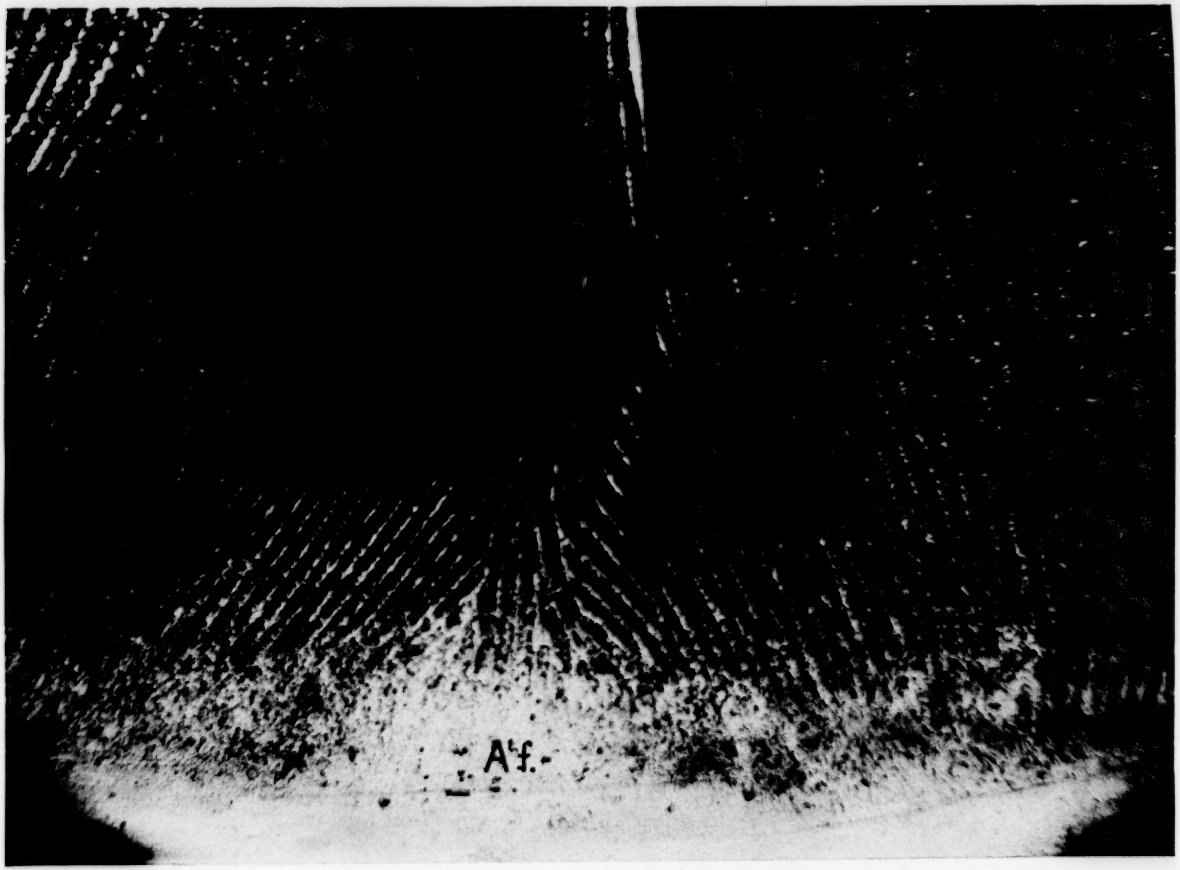

FIG. 12

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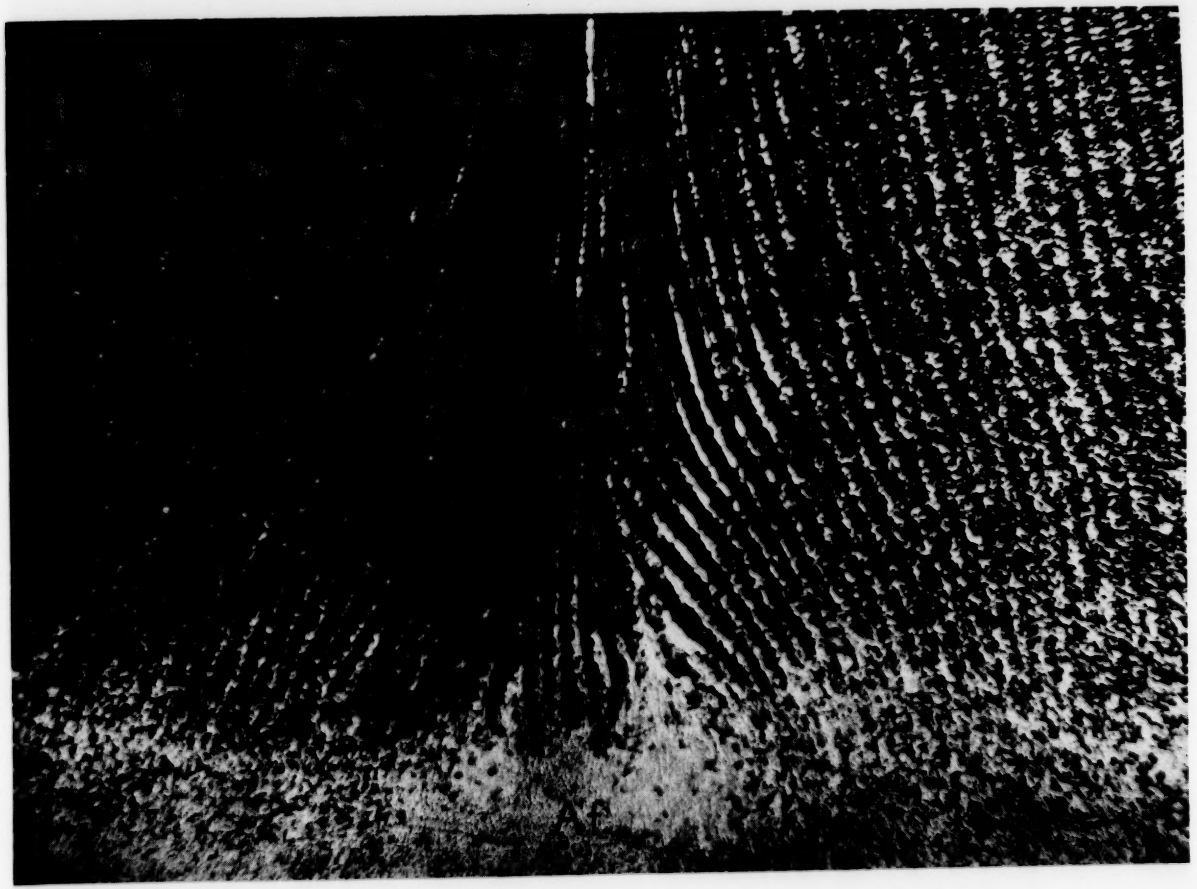

FIG. 13

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PLATE VIII

FIG. 14

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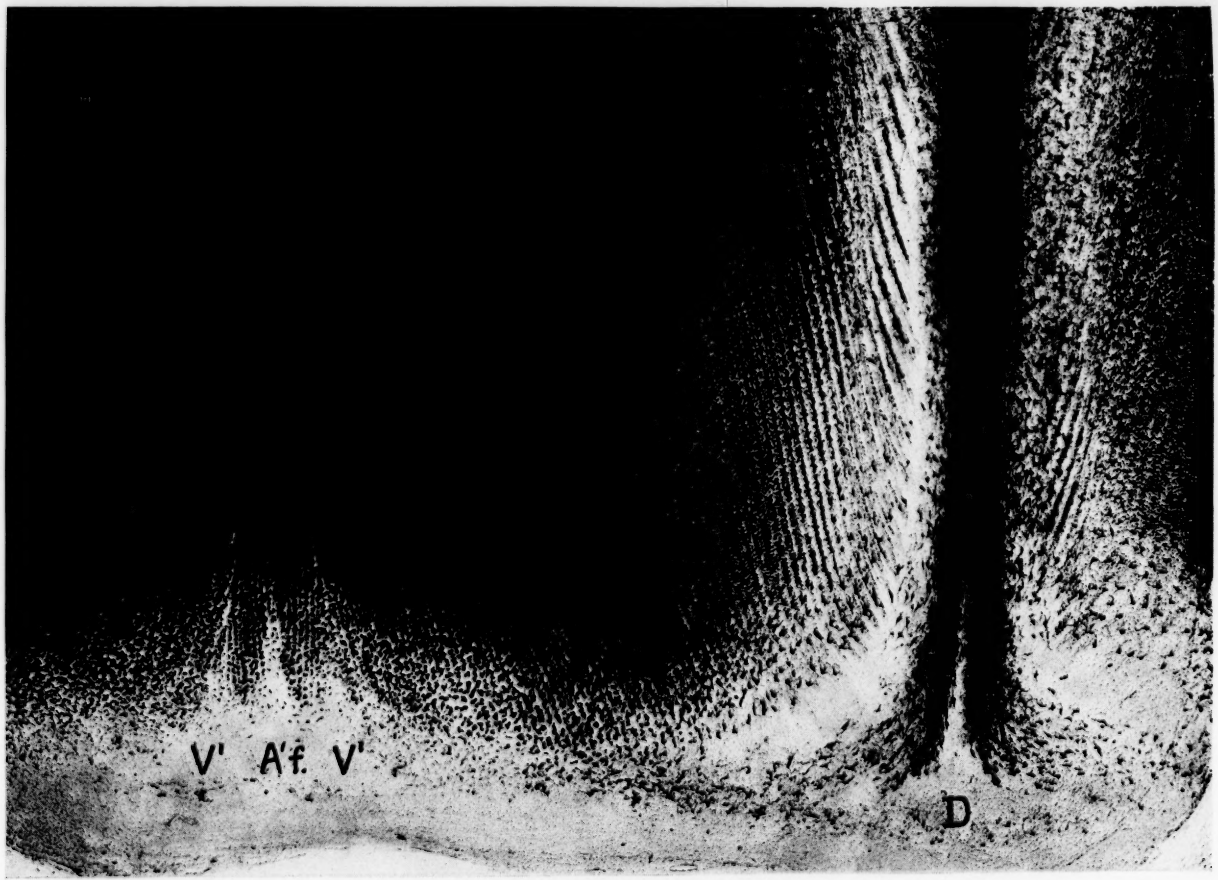

FIG. 15

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PLATE X

FIG. 16

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PLATE XI

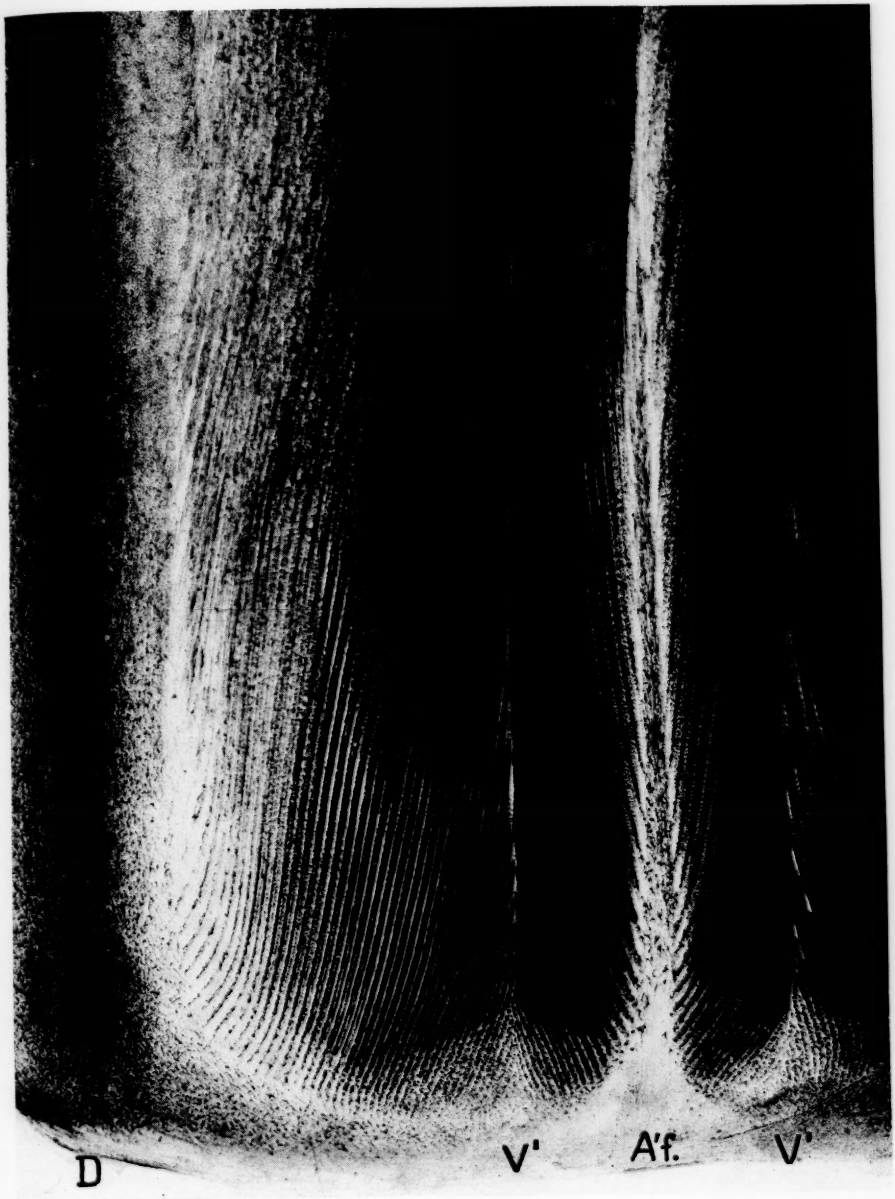

FIG. 17

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PLATE XII

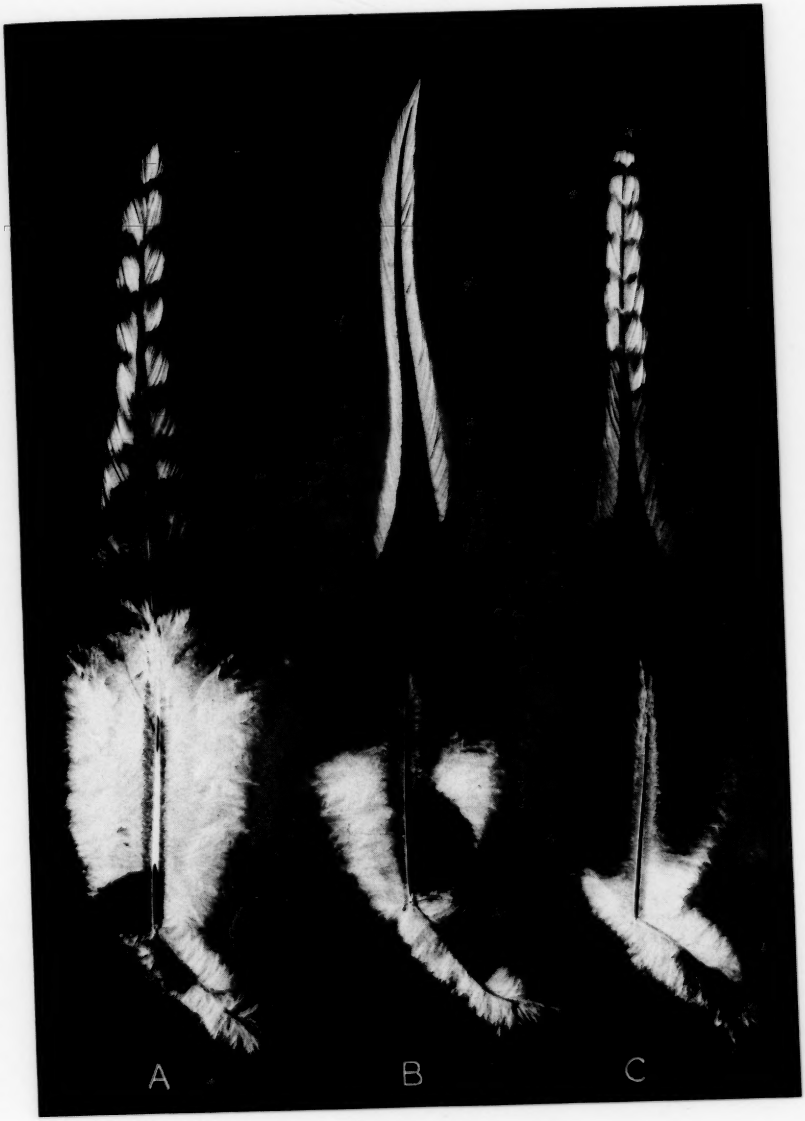

FIG. 18

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Published at the end of January, April, July, and October

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69 Eccleston Square
London, S.W.1., England

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St. Martin's Street
London, W.C.2., England